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(54) Title: REGULATORY SEQUENCES INVOLVED IN HYPOXIA REGULATED GENE EXPRESSION AND USES THEREOF		
(57) Abstract Described are recombinant DNA molecules comprising regulatory sequence(s) of the Vascular Endothelial Growth Factor (VEGF) gene or of a gene homologous to the VEGF gene, being capable of modulating hypoxia inducible expression of a heterologous DNA sequence <i>in vivo</i> . Vectors comprising said recombinant DNA molecules are provided. Also provided are pharmaceutical and diagnostic compositions comprising such recombinant DNA molecules and vectors. Furthermore, cells and transgenic non-human animals, comprising the aforementioned recombinant DNA molecules or vectors stably integrated into their genome and their use for the identification of substances capable of suppressing or activating transcription of a hypoxia inducible gene are described. Described is furthermore the use of the before described recombinant DNA molecules and vectors for the preparation of pharmaceutical compositions for treating, preventing, and/or delaying a vascular or tumorous disease in a subject. Furthermore, uses of the recombinant DNA molecules and vectors of the invention for the preparation of pharmaceutical compositions for inducing a vascular or tumorous disease in a non-human animal are provided. In addition, methods for identifying agonists/activators or antagonists/inhibitors of genes or gene products involved in hypoxia and/or ischemia, compounds identifiable by said method, antibodies directed to said compounds as well as to pharmaceutical and diagnostic compositions comprising said agonists/activators, antagonists/inhibitors and/or antibodies are described. Such compositions may be useful for the treatment or prevention or for diagnosing of vascular diseases.		

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Regulatory sequences involved in hypoxia regulated gene expression and uses thereof

The present invention relates to recombinant DNA molecules comprising regulatory sequence(s) of the Vascular Endothelial Growth Factor (VEGF) gene or of a gene homologous to the VEGF gene, being capable of modulating hypoxia inducible expression of a heterologous DNA sequence *in vivo*. The present invention also provides vectors comprising said recombinant DNA molecules. The present invention additionally relates to pharmaceutical and diagnostic compositions comprising such recombinant DNA molecules and vectors. Furthermore, the present invention relates to cells and transgenic non-human animals, comprising the aforementioned recombinant DNA molecules or vectors stably integrated into their genome and their use for the identification of substances capable of suppressing or activating transcription of a hypoxia inducible gene. The present invention also relates to the use of the before described recombinant DNA molecules and vectors for the preparation of pharmaceutical compositions for treating, preventing, and/or delaying a vascular or tumorous disease in a subject. Furthermore, the recombinant DNA molecules and vectors of the invention can be used for the preparation of pharmaceutical compositions for inducing a vascular or tumorous disease in a non-human animal. In addition, the present invention relates to a method for identifying agonists/activators or antagonists/inhibitors of genes or gene products involved in hypoxia and/or ischemia, to compounds identifiable by said method, to antibodies directed to said compounds as well as to pharmaceutical and diagnostic compositions comprising said agonists/activators, antagonists/inhibitors and/or antibodies. Such compositions may be useful for the treatment or prevention or for diagnosing of vascular diseases.

In the field of neuroscience and medical therapy, there is a great demand for test systems to study the function and interaction of gene products, the malfunction or expression of which cause vascular and/or tumorous diseases. Such systems would also be suitable for drug development against such diseases. A prominent example for such diseases are diseases which develop from cells in tissues that are faced with low oxygen tension under certain physiological (embryogenesis) as well as pathological (tumor growth, wound healing) conditions. The compensatory mechanisms that enable these cells to survive hypoxic conditions involve activation and repression of certain genes (for review see Bunn, *Physiol. Rev.* 76 (1996), 839-885). In order to restore an adequate oxygen supply, either improved oxygen transport or new vessel formation is required. Two key factors involved in these processes are Erythropoietin (EPO) and Vascular Endothelial Growth Factor (VEGF). Hypoxia inducible VEGF gene expression is thus believed to play an important role in the growth of solid tumors which require neovascularization (Plate, *Nature* 359 (1992), 845-847). Neoplasias can be divided into two groups according to their VEGF expression pattern: one group constitutively expresses VEGF in all tumor cells, e.g., VHL-disease associated hemangioblastomas (Wizigmann-Voos, *Cancer Res.* 55 (1995), 1358-1364), whereas the second is characterized by upregulation of VEGF expression in advanced stages of tumor development in a subset of tumor cells. Gliomas, the most malignant human brain tumors, belong to this latter group. Expression of VEGF in these tumors is highly upregulated and restricted to perinecrotic cells (Shweiki, *Nature* 359 (1992), 843-845; Plate, *Int. J. Cancer* 59 (1994), 520-529). Due to their special morphology and location these cells have been designated "perinecrotic palisading" cells (PNP cells) (Russel, *Pathology of tumors of the nervous system*, London: Arnold, 1989). The localization of PNP cells at the border of necrotic foci in the center of the tumor suggests that these cells are subjected to low oxygen partial pressure. Although there is no experimental evidence to date, it is widely assumed that this special microenvironment provides a major stimulus for the increased expression of VEGF in the PNP cells. Data supporting this view came from experiments with multicellular spheroids, a commonly used model for solid tumors. Exposure of cultured cell spheroids to hypoxia leads to the upregulation of VEGF gene expression in the inner cell layers (Shweiki, *Proc. Natl. Acad. Sci. USA* 92 (1995), 768-772). Experimental data have provided evidence that

the genes for EPO and VEGF are regulated by low oxygen tension using similar sensing and control mechanisms (Goldberg, J. Biol. Chem. 269 (1994), 4355-4359). In addition to transcriptional activation, mRNA stabilization is involved in hypoxic regulation of both EPO and VEGF (Goldberg, Blood 77 (1991), 271-277; Schuster, Blood 73 (1989), 13-16; Levy, J. Biol. Chem. 270 (1995), 13333-13340; Levy, J. Biol. Chem. 271 (1996), 2746-2753; Ikeda, J. Biol. Chem. 270 (1995), 19761-19766; Rondon, FEBS Lett. 359 (1995), 267-270). The characterization of the 3' hypoxia enhancer of the EPO gene led to the discovery of a hypoxia-inducible protein complex, termed Hypoxia-inducible factor 1 (HIF 1) (Semenza, Mol. Cell. Biol. 12 (1992), 5447-5454). This transcription factor consists of HIF 1 α and HIF 1 β (ARNT), both members of the bHLH-PAS-domain family (Wang, Proc. Natl. Acad. Sci. USA 92 (1995), 5510-5514). Similarities in the hypoxic induction of EPO and VEGF suggested that VEGF expression induced by low oxygen tension is subject to regulation by the same transcription factor.

Meanwhile, HIF 1 binding and transactivation was reported for the human and rat VEGF genes (Levy, J. Biol. Chem. 270 (1995), 13333-13340; Liu, Circ. Res. 77 (1995), 638-643; Forsythe, Mol. Cell. Biol. 16 (1996), 4604-4613). Results from reporter gene studies employing the rat VEGF promoter led to the conclusion that, in addition to the HIF 1 binding site (TACGTG), other *cis*-acting elements may be involved in transcriptional control of VEGF gene expression by hypoxia (Levy, J. Biol. Chem. 270 (1995), 13333-13340). Forsythe has reported that transcriptional up-regulation of the VEGF gene during hypoxia is dependent upon transactivation by the HIF 1 transcription factor, which binds to a HIF 1 consensus site located in the 5' flanking region of the VEGF gene and that a residual level of hypoxic induction for human VEGF-luciferase constructs lacking the HIF 1 binding site remains (Forsythe, Mol. Cell. Biol. 16 (1996), 4604-4613). On the basis of earlier studies it was hypothesized that a CACACAGC (bp -921 to -912) sequence stretch, highly homologous to the human EPO 3' hypoxia enhancer, is involved in hypoxia induced upregulation of VEGF gene expression. Replacement of this element in the human EPO enhancer by the mouse sequence completely abolished the hypoxia response (Semenza, Mol. Cell. Biol. 12 (1992), 5447-5454). Conversely, in the human VEGF 5' flanking region neither replacement of the motif by the mouse EPO sequence, nor internal deletion, had an effect on hypoxic induction, indicating that this sequence

stretch is not involved in hypoxic upregulation of VEGF gene expression. Furthermore, determinants for the hypoxia-regulated mRNA stability have been localized in the 3' untranslated region of the VEGF mRNA (Levy, J. Biol. Chem. 271 (1996), 2746-2753; Levy, J. Biol. Chem. 271 (1996), 25492-25497; Shima, J. Biol. Chem. 271 (1996), 3877-3883). Recently, a new method has been developed for assessing tissue oxygen partial pressure using EF5, a nitroimidazole whose binding affinity for macromolecules varies according to the cellular oxygen partial pressure. Waleh and coworkers used this method to demonstrate that expression of VEGF colocalizes with regions of low oxygen partial pressure in EMT6 and HT29 multicellular spheroids (Waleh, Cancer Res. 55 (1995), 6222-6226), consistent with the hypothesis that hypoxia can regulate VEGF gene expression. The evidence on the regulation of VEGF gene expression by hypoxia in gliomas has thus far been based on the colocalization of increased VEGF mRNA expression and regions of low oxygen, and on transfection studies performed with VEGF reporter gene constructs using cultured glioma cells *in vitro*. However, it is not known whether indeed the HIF1 binding site is essential and sufficient to confer full hypoxia inducible expression *in vitro* and *in vivo* or whether additional regulatory sequences are required. For studying all aspects of genes involved in both hypoxia regulated physiological and pathological angiogenesis such as in neoplasias and for the development of hypoxia specific drugs, test systems are required which closely resemble hypoxia regulated gene expression *in vivo* since otherwise non-informative or even false positive results may be obtained.

Thus, the technical problem of the present invention is to provide a means that allows studying hypoxia regulated gene expression *in vivo*.

The solution to this technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the invention relates to a recombinant DNA molecule comprising:

- (a) a first regulatory sequence of a promoter active in mammalian cells;
- (b) operatively linked thereto a heterologous DNA sequence; and
- (c) operatively linked thereto a second regulatory sequence derived from the 3'-untranslated region of the Vascular Endothelial Growth factor (VEGF) gene or

of a gene homologous to the VEGF gene being capable of regulating hypoxia inducible expression *in vivo*.

In accordance with the present invention, regulatory sequences involved in hypoxia regulated gene expression have been identified. Said regulatory sequence(s) are suitable to confer hypoxia regulated expression to a heterologous DNA sequence, wherein the regulation of the expression of said heterologous DNA sequence is substantially identical with the hypoxia regulated expression the VEGF gene.

Experiments with stably transfected GS9L cell clones containing mouse VEGF-lacZ reporter gene constructs injected into syngeneic rats were performed in accordance with the present invention. These experiments revealed that although GS9L cells clonally selected to contain the reporter gene were used to produce the transplanted tumors such that all GS9L cells in the tumor should contain the VEGF reporter gene, only the PNP cells upregulated its expression, confirming that the special microenvironment of the PNP cells confers a distinct regulation onto the VEGF gene. VEGF expression in glioma tumors growing in animals is restricted to distinctive perinecrotic palisading (PNP) cells that flank necrotic regions within the tumor. When injected subcutaneously into syngeneic rats, GS9L glioma cells form tumors with distinctive necrotic foci which are histologically similar to human gliomas. *In situ* hybridization revealed high level expression of VEGF mRNA in PNP cells of these experimental tumors (Plate, Cancer Res. 53 (1993), 5822-5827). Furthermore, EF 5 immunostaining for regions of low oxygen partial pressure overlapped with expression of β -galactosidase driven by mouse VEGF regulatory sequences. These results obtained in accordance with the present invention demonstrate that hypoxia is indeed the microenvironmental stimulus for upregulation of VEGF expression in the PNP cells of gliomas and that the 3'-untranslated region of the VEGF gene affects hypoxia regulated gene expression. Thus, in accordance with the present invention, it was shown for the first time that the expression of a heterologous DNA sequence under the control of said regulatory sequence results in an increased level of expression of said heterologous DNA sequence. These results obtained in accordance with the present invention demonstrate that the regulatory sequences described herein can be used to direct hypoxia inducible expression of heterologous DNA sequences.

The recombinant DNA molecule of the invention allows studying the function and interaction of proteins which are expressed and up- or downregulated under hypoxic conditions and which are supposed to be involved in vascular and tumorous diseases. Thus, the regulatory sequences of the invention are particularly suited and useful for the engineering of transgenic cells and non-human animals which can serve as a test system for the development of drugs for the treatment of vascular and tumorous diseases due to hypoxia induced gene expression. The genomic DNA of the VEGF gene comprising the 5' and 3' regulatory sequences can be obtained, for example, by screening a phage library of genomic DNA in the vector λ FixII (Stratagene, La Jolla, CA) generated by conventional methods known in the art.

In the context of the present invention the term "a first regulatory sequence of a promoter active in mammalian cells" means a nucleotide sequence comprising binding sites of transcription factors and/or regulatory proteins.

For the purpose of the present invention the term "a second regulatory sequence derived from the 3'-untranslated region of the VEGF gene" means a nucleotide sequence of the 3'-untranslated sequences from the VEGF gene from mouse capable of regulating hypoxia induced gene expression.

The term "regulatory sequence derived from the 3'-untranslated region of a gene homologous to the VEGF gene" includes regulatory sequences of a gene from another species, for example, humans and other mammals such as rat which is homologous to the VEGF gene of mouse and which confers the same expression pattern. As can be seen from figure 1a and b, which compares the 3' untranslated region of the VEGF gene from mouse with that of human and rat, respectively, the nucleotide sequence of said region is highly conserved. Thus, it is to be expected that the 3' untranslated regions of the human and the rat VEGF gene function in the same fashion as that of the mouse gene does. Such regulatory sequences are characterized by their capability of conferring hypoxia regulated expression of a heterologous DNA sequence *in vivo*, preferably in glioma cells. Thus, according to the present invention, regulatory sequences from other species can be used that are functionally homologous to the regulatory sequences of the 3'-untranslated region of the VEGF gene from mouse, or regulatory sequences of genes that display an

identical pattern of expression, in the sense of being expressed under hypoxic conditions, preferably in glioma cells.

It is possible for the person skilled in the art to isolate by employing the known VEGF gene from mouse, corresponding genes from other species, for example, humans and other mammals. This can be done by conventional techniques known in the art, for example, by using VEGF gene sequences as a hybridization probe or by designing appropriate PCR primers. It is then possible to isolate the corresponding regulatory sequences by conventional techniques and test them for their expression pattern. For this purpose, it is, for instance, possible to fuse the regulatory sequences to a reporter gene, such as the luciferase or green fluorescent protein (GFP) encoding genes and assess the expression of the reporter gene in transgenic animals, for example in mice. The nucleotide sequence of the human and rat VEGF genes including the 3'-UTR (UTR = untranslated region) are described in Levy, J. Biol. Chem. 271 (1996), 25492-25497 and 2746-2753. Furthermore, the nucleotide sequence of the 3'-UTR of the human VEGF gene can be obtained from the GenBank data base (accession no. Y08736).

The present invention also relates to recombinant DNA molecules comprising regulatory sequences which are substantially identical to that of the VEGF 3'-UTR or to a 3'-UTR of a homologous gene or to fragments thereof and which are able to contribute to hypoxia regulated expression *in vivo* in mice or other mammals.

Such regulatory sequences differ at one or more positions from the above-mentioned regulatory sequences but still have the same specificity, namely they comprise the same or similar sequence motifs responsible for the above described expression pattern. Preferably such regulatory sequences hybridize to one of the above-mentioned regulatory sequences, most preferably under stringent conditions. Particularly preferred are regulatory sequences which share at least 85%, more preferably 90-95%, and most preferably 96-99% sequence identity with one of the above-mentioned regulatory sequences and have the regulatory properties as described above. Such regulatory sequences also comprise those which are analogues or are derivatives, and differ, for example by way of nucleotide deletion(s), insertion(s), substitution(s), addition(s), and/or recombination(s) and/or any other modification(s) known in the art, either alone or in combination from the above-described nucleotide sequence. Methods for introducing such modifications in the

nucleotide sequence of the regulatory sequences of the invention are well known to the person skilled in the art and described, for example, in Sambrook (Molecular cloning; A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY (1989)). All such fragments, analogues and derivatives of the regulatory sequence of the invention are included within the scope of the present invention, as long as the essential characteristic regulatory properties as defined above remain unaffected in kind. It is also immediately evident to the person skilled in the art that further regulatory sequences may be added to the regulatory sequences of the invention. For example promoters per se, transcriptional enhancers and/or sequences which allow for induced expression of the regulatory sequences of the invention may be employed. A suitable inducible system is for example tetracycline-regulated gene expression which is described by, e.g., Gossen (Proc. Natl. Acad. Sci. USA 89 (1992), 5547-5551; Trends Biotech. 12 (1994), 58-62).

Further regulatory sequences comprise sequences which influence the specificity and/or level of expression, for example in the sense that they confer cell and/or tissue specificity or developmentally and/or inducibly regulated gene expression. Such sequences can be located upstream of or comprising the transcription initiation site, such as a promoter, but can also be located downstream thereof, e.g., in transcribed but untranslated leader sequences.

The term "promoter" refers to the nucleotide sequences necessary for transcription initiation, i.e. RNA polymerase binding, and also includes, for example, the TATA box.

The term "*in vivo*" for the purpose of the present invention is used for cells in an organism as opposed to cells growing in culture (*in vitro*).

The term "heterologous" with respect to the DNA sequence being operatively linked to the promoter of the invention means that said DNA sequence is not naturally linked to the regulatory sequences comprised in the recombinant DNA molecule of the invention.

In a preferred embodiment of the invention, said second regulatory sequence is selected from the group consisting of

(a) DNA sequences comprising a nucleotide sequence as given in SEQ ID NO. 1;

- (b) DNA sequences comprising the nucleotide sequence of the 3'-untranslated region from the human or rat VEGF gene;
- (c) DNA sequences comprising a nucleotide sequence which hybridizes with a nucleotide sequence of (a) or (b) under stringent conditions and which is capable of regulating hypoxia induced expression *in vivo*;
- (d) DNA sequences comprising a nucleotide sequence which is conserved in the nucleotide sequences of (a) and (b); and
- (e) DNA sequences comprising a fragment, analogue or derivative of a nucleotide sequence of any one of (a) to (d) capable of regulating hypoxia induced expression *in vivo*.

In another preferred embodiment said first regulatory sequence of the invention comprises an AP-1 binding site, an SP1 binding site, or a Hypoxia Inducible Factor (HIF)1 binding site or any combination(s) thereof. Most preferably, said first regulatory sequence comprises an AP-1 and an HIF1 binding site.

In a further preferred embodiment of the invention said first regulatory sequence is derived from a promoter of hypoxia inducible genes, genes encoding growth factors or their receptors or glycolytic enzymes. Preferably said growth factor is VEGF, PDGF or Fibroblast growth factor.

Expression comprises transcription of the DNA sequence, preferably into a translatable mRNA. Regulatory elements ensuring expression in eukaryotic cells, preferably mammalian cells, are well known to those skilled in the art. They usually comprise promoters ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Additional regulatory elements may include transcriptional as well as translational enhancers. Preferably said further regulatory sequence is a minimal promoter, preferably derived from SV40. These promoters can be combined with the regulatory sequences of the invention in order to mediate hypoxia inducible gene expression of heterologous DNA sequences.

In accordance with the present invention it has been surprisingly found that hypoxia mediated gene expression is not confined to one particular region of the VEGF gene but that regulatory sequences in the promoter and in the 3' untranslated region of the VEGF gene contribute to hypoxia mediated expression. Moreover, the results obtained in accordance with the present invention revealed that when both, the regulatory sequence of the VEGF promoter and that of the 3' untranslated region of the gene, are operatively linked to a heterologous DNA sequence (in this case a reporter gene) this leads to an unexpected synergistic effect in that a significant up-regulation of the expression of the heterologous DNA sequence is achieved under hypoxic or ischemic conditions.

Thus, in a particularly preferred embodiment said first regulatory sequence comprises a DNA sequence selected from the group consisting of

- (a) DNA sequences comprising the nucleotide sequence as given in SEQ ID NO. 2, preferably from nucleotide 1 to nucleotide 280;
- (b) DNA sequences comprising the nucleotide sequence of the human or rat VEGF promoter;
- (c) DNA sequences comprising a nucleotide sequence which hybridizes with a nucleotide sequence of (a) or (b) under stringent conditions and which is capable of regulating hypoxia induced expression *in vivo*;
- (d) DNA sequences comprising a nucleotide sequence which is conserved in the nucleotide sequences of (a) and (b); and
- (e) DNA sequences comprising a fragment, analogue or derivative of a nucleotide sequence of any one of (a) to (d) capable of regulating hypoxia inducible expression *in vivo*.

In a previous study (Ikeda, J. Biol. Chem. 270 (1995), 19761-19766) the hypoxia responsiveness of the human VEGF promoter in C6 glioma cells was attributed to a 288bp SacI-BanI fragment, located 1176 bp to 888 bp upstream of the transcription initiation site. Experiments performed in accordance with the present invention revealed, however, that the HIF 1 binding site is not sufficient to confer full hypoxia inducibility to the reporter gene in C6 glioma cells. The results obtained in accordance with the invention indicate that sequences upstream of the HIF 1

consensus site potentiate the hypoxic induction mediated by HIF 1. By employing site directed mutagenesis, it was shown that this potentiating effect is surprisingly due to an AP 1 binding site homologous to that found in the promoter of the anoxia inducible VL30 retrotransposon, which has been described recently (Estes, Exp. Cell Res. 220 (1995), 47-54). AP 1 could be demonstrated to interact with this *cis*-element under both normoxic and hypoxic conditions and constitutive binding of the AP 1 complex to the "potentiating" *cis*-element under both normoxic and hypoxic conditions is consistent with the observation that the -1176/-1015 fragment is unable to confer hypoxia-responsiveness on its own in reporter assays. These results obtained in accordance with the present invention suggest that a complex cell-type specific regulation of VEGF gene expression occurs under hypoxic conditions. Although AP 1 has been shown to be hypoxia-inducible in both its DNA-binding and transactivation capacities in, e.g., HeLa (Ruppec, Eur. J. Biochem. 234 (1995), 632-640 and Hep3B (Prabhakar, Brain Res. 697 (1995), 266-270) cells, no alterations in AP 1 function could be shown in other cell lines (Prabhakar, Brain Res. 697 (1995), 266-270). Thus, AP 1-mediated modulation of gene expression in response to hypoxia may occur in a cell-type specific manner. Taken together the results obtained in accordance with the present invention have shown that the binding consensus for HIF 1 is crucial for hypoxia induced transcriptional activation of the VEGF gene. The complete loss of inducibility after internal deletion of this *cis*-element, however, is a novel finding and is in contrast to the results previously published for rat and human VEGF genes in PC 12 and Hep3B cells, respectively (Levy, J. Biol. Chem. 270 (1995), 13333-13340; Forsythe, Mol. Cell. Biol. 16 (1996), 4604-4613). Using mutations or deletions of the HIF 1 binding consensus both authors found residual inducibility of a reporter gene *in vitro*. Furthermore, in contrast to the expectations and interpretations of the prior art, upstream sequences have a potentiating effect on hypoxia induction, which can be attributed to binding sites of transcription factors such as for AP1. This effect may be part of a cell-type specific modulation pathway allowing the fine-tuning of the transcriptional response to hypoxia.

In a further preferred embodiment, the heterologous DNA sequence of the above-described recombinant DNA molecules encodes a peptide, protein, antisense RNA,

sense RNA and/or ribozyme. The recombinant DNA molecule or vector of the invention can be used alone or as part of a vector to express heterologous DNA sequences, which, e.g., encode proteins other than VEGF, in cells of the blood vessel wall, i.e. endothelial cells, or in neuronal cells, for, e.g., gene therapy or diagnostics of vascular diseases such as atherosclerosis or determination of the oxygen tension of a cell, tissue or organ. The recombinant DNA molecule or vector containing DNA sequence encoding a protein of interest is introduced into the cells which in turn produce the protein of interest, preferably upon hypoxia induction. For example, sequences encoding t-PA (Pennica, Nature 301 (1982), 214), p21 cell cycle inhibitor (El-Deiry, Cell 75 (1993), 817-823), or nitric oxide synthase (Bredt, Nature 347 (1990), 768-770) may be operatively linked to the regulatory sequences of the invention and expressed in the cells. For example, thrombolytic agents can be expressed under the control of the regulatory sequences of the invention for expression by vascular endothelial cells in blood vessels, e.g., vessels occluded by aberrant blood clots under hypoxic conditions. Other heterologous proteins, e.g., proteins which inhibit smooth muscle cell proliferation, e.g., interferon- γ and atrial natriuretic polypeptide, may be specifically expressed in cells in the hypoxic state to ensure the delivery of these therapeutic peptides to an atherosclerotic lesion or an area at risk of developing an atherosclerotic lesion, e.g., an injured blood vessel.

The hypoxic regulatory sequences of the invention may also be used in gene therapy to promote angiogenesis to treat diseases such as peripheral vascular disease or coronary artery disease (Isner, Circulation 91 (1995), 2687-2692). For example, the regulatory sequences of the invention can be operatively linked to sequences encoding cellular growth factors which promote angiogenesis, e.g., VEGF, acidic fibroblast growth factor, basic fibroblast growth factor and the like.

In a particularly preferred embodiment of the present invention, said protein is selected from the group consisting of Hypoxia Inducible Factor (HIF), HIF-Related Factor (HRF), tissue plasminogen activator, p21 cell cycle inhibitor, nitric oxide synthase, interferon- γ , atrial natriuretic polypeptide, p53, proteins encoded by apoptosis inducing genes of the bcl2 family, and monocyte chemotactic proteins.

In another particularly preferred embodiment of the invention, said protein is a scorable marker, preferably luciferase, green fluorescent protein or β -galactosidase. This embodiment is particularly useful for simple and rapid screening methods for compounds and substances described herein below capable of modulating the hypoxia regulated gene expression. For example, glioma cells can be cultured in the presence and absence of the candidate compound in order to determine whether the compound affects the expression of genes which are under the control of regulatory sequences of the invention, which can be measured, e.g., by monitoring the expression of the above-mentioned marker. It is also immediately evident to those skilled in the art that other marker genes may be employed as well, encoding, for example, selectable marker which provide for the direct selection of compounds which induce or inhibit the expression of said marker.

The regulatory sequences of the invention may also be used in methods of antisense therapy. Antisense therapy may be carried out by administering to an animal or a human patient, a recombinant DNA containing the regulatory sequences of the invention operably linked to a DNA sequence, i.e. an antisense template which is transcribed into an antisense RNA. The antisense RNA may be a short (generally at least 10, preferably at least 14 nucleotides, and optionally up to 100 or more nucleotides) nucleotide sequence formulated to be complementary to a portion of a specific mRNA sequence. Standard methods relating to antisense technology have been described (Melani, Cancer Res. 51 (1991), 2897-2901). Following transcription of the DNA sequence into antisense RNA, the antisense RNA binds to its target mRNA molecules within a cell, thereby inhibiting translation of the mRNA and down-regulating expression of the protein encoded by the mRNA. For example, an antisense sequence complementary to a portion of or all of the VEGF mRNA (Terman, Oncogene 6 (1991), 1677-1683) would inhibit the expression of VEGF, which in turn would inhibit angiogenesis. Such antisense therapy may be used to treat cancer, particularly to inhibit angiogenesis at the site of a solid tumor, as well as other pathogenic conditions which are caused by or exacerbated by angiogenesis, e.g., inflammatory diseases such as rheumatoid arthritis, and diabetic retinopathy.

The expression of other proteins involved in cell proliferation and angiogenesis may also be inhibited in a similar manner, for example, cell cycle proteins (thereby

inhibiting cell proliferation, and therefore, angiogenesis); coagulation factors such as von Willebrand factor; and cell adhesion factors, such as ICAM-1 and VCAM-1 (Bennett, J. Immunol. 152 (1994), 3530-3540).

Thus, in a further preferred embodiment of the present invention, said antisense RNA or said ribozyme are directed against a gene involved in vasculogenesis and/or angiogenesis and/or tumors which require neovascularization.

In a further embodiment, the invention relates to nucleic acid molecules of at least 15 nucleotides in length hybridizing specifically with a regulatory sequence as described above or with a complementary strand thereof. Specific hybridization occurs preferably under stringent conditions and implies no or very little cross-hybridization with nucleotide sequences having no or substantially different regulatory properties. Such nucleic acid molecules may be used as probes and/or for the control of gene expression. Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary in length. Preferred are nucleic acid probes of 17 to 35 nucleotides in length. Of course, it may also be appropriate to use nucleic acids of up to 100 and more nucleotides in length. The nucleic acid probes of the invention are useful for various applications. On the one hand, they may be used as PCR primers for amplification of regulatory sequences according to the invention. Another application is the use as a hybridization probe to identify regulatory sequences hybridizing to the regulatory sequences of the invention by homology screening of genomic DNA libraries. Nucleic acid molecules according to this preferred embodiment of the invention which are complementary to a regulatory sequence as described above may also be used for repression of expression of a gene comprising such regulatory sequences, for example due to an antisense or triple helix effect or for the construction of appropriate ribozymes (see, e.g., EP-B1 0 291 533, EP-A1 0 321 201, EP-A2 0 360 257) which specifically cleave the (pre)-mRNA of a gene comprising a regulatory sequence of the invention. Selection of appropriate target sites and corresponding ribozymes can be done as described for example in Steinecke, Ribozymes, Methods in Cell Biology 50, Galbraith et al. eds Academic Press, Inc. (1995), 449-460. Furthermore, the person skilled in the art is well aware that it is also possible to label such a nucleic acid probe with an appropriate marker for specific applications, such as for the detection of the

presence of a nucleic acid molecule of the invention in a sample derived from an organism.

The above described nucleic acid molecules may either be DNA or RNA or a hybrid thereof. Furthermore, said nucleic acid molecule may contain, for example, thioester bonds and/or nucleotide analogues, commonly used in oligonucleotide anti-sense approaches. Said modifications may be useful for the stabilization of the nucleic acid molecule against endo- and/or exonucleases in the cell. Said nucleic acid molecules may be transcribed by an appropriate vector containing a chimeric gene which allows for the transcription of said nucleic acid molecule in the cell. Such nucleic acid molecules may further contain ribozyme sequences which specifically cleave the (pre)-mRNA comprising the regulatory sequence of the invention. Furthermore, oligonucleotides can be designed which are complementary to a regulatory sequence of the invention (triple helix; see Lee, Nucl. Acids Res. 6 (1979), 3073; Cooney, Science 241 (1988), 456 and Dervan, Science 251 (1991), 1360), thereby preventing transcription and the production of the encoded protein.

The present invention also relates to vectors, particularly plasmids, cosmids, viruses and bacteriophages used conventionally in genetic engineering that comprise a recombinant DNA molecule of the invention. Preferably, said vector is an expression vector and/or a targeting vector. Expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of the recombinant DNA molecule or vector of the invention into targeted cell population. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors; see, for example, the techniques described in Sambrook, Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, the recombinant DNA molecules and vectors of the invention can be reconstituted into liposomes for delivery to target cells.

The present invention furthermore relates to host cells transformed with a DNA molecule or vector of the invention. Said host cell may be a prokaryotic or eukaryotic cell. The vector or recombinant DNA molecule of the invention which is present in the

host cell may either be integrated into the genome of the host cell or it may be maintained extrachromosomally. In this respect, it is also to be understood that the recombinant DNA molecule of the invention can be used for "gene targeting" and/or "gene replacement", for restoring a mutant gene or for creating a mutant gene via homologous recombination.

The host cell can be any prokaryotic or eukaryotic cell, such as a bacterial, insect, fungal, plant, animal or human cell. Preferred fungal cells are, for example, those of the genus *Saccharomyces*, in particular those of the species *S. cerevisiae*. Suitable mammalian cell lines comprise Saos-2 human osteosarcoma cells (ATCC HTB-85), HeLa human epidermoid carcinoma cells (ATCC CRL-7923), HepG2 human hepatoma cells (ATCC HB-8065), human fibroblasts (ATCC CRL-1634), U937 human histiocytic lymphoma cells (ATCC CRL-7939), RD human embryonic rhabdomyosarcoma cells (ATCC CCL-136), MCF7 human breast adenocarcinoma cells (ATCC HTB-22), JEG-3 human choriocarcinoma cells (ATCC HB36), A7r5 fetal rat aortic smooth muscle cells (ATCC CRL-1444), NIH 3T3 mouse fibroblasts (ATCC CRL-1658) HEP 3B (ATCC HB 8064), C6 (ATCC CCL 107) and GS 9L obtainable from the American Type Culture Collection. Primary-culture HUVEC may be obtained from Clonetics Corp. (San Diego, CA) and can be grown in EGM medium containing 2% fetal calf serum (Clonetics). Primary-culture human aortic and intestinal smooth muscle cells can also be obtained from Clonetics Corp. Most preferably said host cell is a glioma cell or derived therefrom, or a primary cell, tumor cell, spheroid cell, aggregate cell, stem cell or a differentiated cell although any other animal, preferably mammalian cell may be appropriate as well.

Moreover, the present invention relates to a pharmaceutical composition comprising at least one of the aforementioned recombinant DNA molecules or vectors of the invention, either alone or in combination, and optionally a pharmaceutically acceptable carrier or excipient. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. Administration of the suitable compositions may be

effected by different ways, e.g., by intravenous, intraperitoneal, subcutaneous, intramuscular, topical or intradermal administration. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosages will vary but a preferred dosage for intravenous administration of DNA is from approximately 10^6 to 10^{22} copies of the DNA molecule. The compositions of the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously; DNA may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery.

It is envisaged by the present invention that the various recombinant DNA molecules and vectors of the invention are administered either alone or in any combination using standard vectors and/or gene delivery systems, and optionally together with an appropriate compound, for example VEGF, and/or together with a pharmaceutically acceptable carrier or excipient. Subsequent to administration, said recombinant DNA molecules may be stably integrated into the genome of the mammal. On the other hand, viral vectors may be used which are specific for certain cells or tissues, preferably for the endothelium and persist in said cells. Suitable pharmaceutical carriers and excipients are well known in the art. The pharmaceutical compositions prepared according to the invention can be used for the prevention or treatment or delaying of different kinds of diseases, which are related to the expression or overexpression of hypoxia regulated genes.

Furthermore, it is possible to use a pharmaceutical composition of the invention which comprises a recombinant DNA molecule or vector of the invention in gene therapy. Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes viruses, retroviruses, adenoviruses, and adeno-associated viruses, among others. Delivery of nucleic acids to a specific site in the body for gene therapy or antisense therapy may also be

accomplished using a biolistic delivery system, such as that described by Williams (Proc. Natl. Acad. Sci. USA 88 (1991), 2726-2729).

Standard methods for transfecting cells with recombinant DNA are well known to those skilled in the art of molecular biology, see, e.g., WO 94/29469. Gene therapy and antisense therapy to prevent or decrease the development of atherosclerosis or inhibit angiogenesis may be carried out by directly administering the recombinant DNA molecule or vector of the invention to a patient or by transfecting endothelial cells with the recombinant DNA molecule or vector of the invention *ex vivo* and infusing the transfected cells into the patient. Furthermore, research pertaining to gene transfer into cells of the germ line is one of the fastest growing fields in reproductive biology. Gene therapy, which is based on introducing therapeutic genes into cells by *ex-vivo* or *in-vivo* techniques is one of the most important applications of gene transfer. Suitable vectors and methods for *in-vitro* or *in-vivo* gene therapy are described in the literature and are known to the person skilled in the art; see, e.g., WO94/29469, WO 97/00957 or Schaper (Current Opinion in Biotechnology 7 (1996), 635-640) and references cited therein. The DNA molecules and vectors comprised in the pharmaceutical composition of the invention may be designed for direct introduction or for introduction via liposomes, or viral vectors (e.g. adenoviral, retroviral) containing said recombinant DNA molecule into the cell. Preferably, said cell is a germ line cell, embryonic cell, or egg cell or derived therefrom. The pharmaceutical compositions according to the invention can be used for the treatment of diseases hitherto unknown as being related to hypoxia regulated gene expression.

It is to be understood that the introduced recombinant DNA molecules and vectors of the invention express the heterologous DNA sequence after introduction into said cell and preferably remain in this status during the lifetime of said cell. For example, cell lines which stably express the heterologous DNA under the control of the regulatory sequence of the invention may be engineered according to methods well known to those skilled in the art. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the recombinant DNA molecule or vector of the invention and a selectable marker, either on the same or separate vectors. Following the introduction of foreign DNA, engineered cells may be

allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows for the selection of cells having stably integrated the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the heterologous DNA sequence under the control of the regulatory sequence of the invention, and which respond to VEGF and/or hypoxia mediated signal transduction. Such engineered cell lines are particularly useful in screening compounds capable of modulating hypoxia mediated gene expression.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, Cell 11(1977), 223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska, Proc. Natl. Acad. Sci. USA 48 (1962), 2026), and adenine phosphoribosyltransferase (Lowy, Cell 22 (1980), 817) in tk⁻, hgprt⁻ or aprt⁻ cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, Proc. Natl. Acad. Sci. USA 77 (1980), 3567; O'Hare, Proc. Natl. Acad. Sci. USA 78 (1981), 1527), gpt, which confers resistance to mycophenolic acid (Mulligan, Proc. Natl. Acad. Sci. USA 78 (1981), 2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, J. Mol. Biol. 150 (1981), 1); hygromycin (Santerre, Gene 30 (1984), 147); or puromycin (pat, puromycin N-acetyl transferase). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.). On the other hand, the person skilled in the art may also use the regulatory sequences of the invention to "knock out" an endogenous gene comprising identical or similar regulatory sequences, for example, by gene targeting, cosuppression, triple helix, antisense or ribozyme technology.

The present invention also relates to compositions comprising at least one of the aforementioned recombinant DNA molecules or vectors, and in the case of diagnostic compositions, optionally suitable means for detection. Said compositions may further contain compounds such as further plasmids, antibiotics and the like for screening transgenic animals and/or animal cells useful for the genetic engineering of non-human animals, preferably mammals and most preferably mouse. The diagnostic compositions of the invention may be used for methods of detecting and isolating regulatory sequences which are a functionally equivalent to regulatory sequences of the invention capable of modulating hypoxia inducible gene expression.

The present invention also relates to a method for the production of a transgenic animal, preferably transgenic mouse, comprising introduction of a recombinant DNA molecule or vector of the invention into a germ cell, an embryonic cell or an egg or a cell derived therefrom. The non-human animal to be used in the method of the invention may be a non-transgenic healthy animal, or may have a disease or disorder, preferably a disease or disorder which is dependent on neovascularization, such as a solid tumor, retinopathy, arthritis or psoriasis. Said disease or disorder may be an inborn insufficiency or naturally developed or caused by genetic engineering, for instance by the expression of a DNA sequence encoding a protein involved in neuronal development and/or diseases as described above, preferably under the control of the regulatory sequence of the invention.

The invention also relates to transgenic non-human animals such as transgenic rats, hamsters, dogs, monkeys, rabbits or pigs comprising a recombinant DNA molecule or vector of the invention or obtained by the method described above, preferably wherein said recombinant DNA molecule is stably integrated into the genome of said non-human animal, preferably such that the presence of said recombinant DNA molecule or vector leads to the transcription and/or expression of the heterologous DNA sequence by the regulatory sequence of the invention.

With the regulatory sequences of the invention, it is now possible to study *in vivo* hypoxia regulated gene expression. Furthermore, since hypoxia induced gene

expression has different functions in different stages of physiological and pathological conditions, it is now possible to determine further regulatory sequences which may be important for the up- or down-regulation of hypoxia inducible genes, for example in specific tumors. In addition, it is now possible to *in vivo* study mutations which affect different functional or regulatory aspects of hypoxia regulated gene expression.

The *in vivo* studies referred to above will be suitable to further broaden the knowledge on the mechanisms involved in hypoxia and/or ischemia. To date, it is known that deprivation of oxygen and nutrients leads to organ and tissue ischemia followed by cell death and necroses. Organs which depend on postmitotic cells for proper function (e.g. cardiomyocytes in the heart and neurons in the brain) are irreversibly destroyed by long lasting ischemia leading to infarction and stroke. Blood vessels occluded by atherosclerotic processes or thrombi cause these life threatening diseases. The self-defense and reparative processes of the body involving new blood vessel (collateral) formation, vessel dilatation and plaque removal are too slow to protect cardiomyocyte and neuronal cell death. Heart cardiac infarction, stroke and peripheral artery disease are the most common diseases of the Western world. The functional integrity and formation (angiogenesis) of blood vessels is regulated by tissue hormones and growth factors which themselves are activated by local hypoxia, ischemia or injury. Short periods of experimental ischemia which by themselves have no detrimental effects in organs such as the heart have been shown to have beneficial effects. This effect is known as preconditioning. As has been demonstrated in accordance with the present invention the regulating sequences described herein account for hypoxia inducible upregulation of a heterologous DNA sequence (here reporter gene) involved in the regulation of hypoxia and ischemia, and thus in the preconditioning response. Thus, recombinant DNA molecule comprising a readout system operatively linked to at least one regulatory sequence capable of mediating or regulating hypoxia and/or ischemia inducible expression of said readout system, preferably the recombinant DNA molecules of the invention can be used to identify new lead compounds which trigger the preconditioning response or support the survival functions of the organ in the

absence of hypoxia/ischemia prior to an anticipated life-threatening infarction or stroke.

Accordingly, the present invention further relates to a method for the identification of an agonist/activator and antagonist/inhibitor of genes or gene products involved in hypoxia and/or ischemia comprising the steps of:

- (a) providing an animal or human cell or tissue, or a non-human animal comprising a recombinant DNA molecule comprising a readout system operatively linked to at least one regulatory sequence capable of mediating or regulating hypoxia and/or ischemia inducible expression of said readout system, wherein said regulatory sequence is preferably a regulatory sequence of the invention;
- (b) culturing said animal or human cell, or tissue or non-human animal in the presence of a sample comprising a plurality of compounds under conditions which permit inducible expression of said readout system;
- (c) identifying a sample and compound, respectively, which leads to suppression or activation and/or enhancement of expression of said readout system in said animal or human cell, or tissue, a non-human animal.

The term "read out system" in context with the present invention means a DNA sequence which upon transcription and/or expression in a cell, tissue or organism provides for a scorable and/or selectable phenotype. Such read out systems are well known to those skilled in the art and comprise, for example, recombinant DNA molecules as described above.

The term "plurality of compounds" in a method of the invention is to be understood as a plurality of substances which may or may not be identical.

Said plurality of compounds may be comprised in, for example, samples, e.g., cell extracts from, e.g., plants, animals or microorganisms. Furthermore, said compounds may be known in the art but hitherto not known to be capable of suppressing or activating and/or enhancing the transcription of a hypoxia inducible gene. The plurality of compounds may be, e.g., added to the culture medium or injected into the animals.

If a sample containing a plurality of compounds is identified in the method of the invention, then it is either possible to isolate the compound from the original sample identified as containing the compound capable of suppressing or activating and/or

enhancing the transcription of a hypoxia regulated gene, or one can further subdivide the original sample, for example, if it consists of a plurality of different compounds, so as to reduce the number of different substances per sample and repeat the method with the subdivisions of the original sample. It can then be determined whether said sample or compound mimics or suppresses the cellular effects of hypoxia. Depending on the complexity of the samples, the steps described above can be performed several times, preferably until the sample identified according to the method of the invention only comprises a limited number of or only one substance(s). Preferably said sample comprises substances of similar chemical and/or physical properties, and most preferably said substances are identical.

In a preferred embodiment of the method of the invention said recombinant DNA molecule comprising said read out system is a recombinant DNA molecule as described in the embodiments hereinbefore.

In a further preferred embodiment of the method of the invention said animal or human cell, tissue or non-human animal is a cell, tissue or transgenic non-human animal described in the embodiments hereinbefore.

In a particularly preferred embodiment of the method of the invention said recombinant DNA molecule comprised in said animal or human cell, tissue or non-human transgenic animal is introduced into the genome by transfection, transformation, electroporation, infection or particle bombardment.

Determining whether a compound is capable of suppressing or activating and/or enhancing the transcription of a hypoxia regulated gene can be done, for example, in mice by monitoring the reporter gene. It can further be done by monitoring the behavior of the transgenic non-human animals of the invention contacted with the compounds and compare it to that of wild-type animals. In an additional embodiment, said behavior may be compared to that of a transgenic non-human animal contacted with a compound which is either known to be capable or incapable of suppressing or activating and/or enhancing the transcription of a hypoxia regulated gene of said transgenic non-human animal of the invention. Furthermore, the person skilled in the

art can monitor the physical behavior. The compounds identified according to the method of the invention are expected to be very beneficial since for the treatment of heart ischemia and peripheral artery diseases so far only approaches using bolus injections or infusions of recombinant VEGF or, alternatively, gene therapy have been used and there is only limited success due to the high turnover of the protein or limited expressivity of transduced genes. In summary, the present invention provides methods for identifying compounds which modulate hypoxia inducible gene expression. For example, activators of hypoxia inducible expression may be used in processes such as wound healing; in contrast, antagonists of expression may be used in the treatment of tumors that rely on vascularization for growth. Compounds found to downregulate expression of a VEGF gene can be used in methods to inhibit angiogenesis, while compounds found to enhance hypoxia mediated expression can be used in methods to promote angiogenesis, for example, to promote wound healing (e.g., healing of broken bones, burns, diabetic ulcers, and traumatic or surgical wounds) or to treat peripheral vascular disease, atherosclerosis, cerebral vascular disease, hypoxic tissue damage (e.g., hypoxic damage to heart tissue), diabetic pathologies such as chronic skin lesions, or coronary vascular disease. These compounds can also be used to treat patients who have, or have had, transient ischemic attacks, vascular graft surgery, balloon angioplasty, frostbite, gangrene, or poor circulation. Compounds identified as having the desired effect (i.e. modulating hypoxia inducible expression) can be tested further in appropriate models of angiogenesis which are known to those skilled in the art.

In a preferred embodiment of the method of the invention the induction of hypoxia is performed by modulation of O₂ partial pressure, or conferred by a compound capable of mimicking the oxygen-sensing and signal transducing mechanism in a cell, preferably cobaltous chloride, desferrioxamine, glucose and nutrient deprivation. Preferably, said compound used for inducing mimicking effects of hypoxia is a hypoxia-inducible factor (HIF) or a HIF-related factor (HRF) or a nucleic acid molecule encoding either of said factors operatively linked to regulatory elements allowing expression of said nucleic acid molecules in said mammalian cell.

The compounds identified or obtained according to the method of the present invention are to be expected very useful in diagnostic and therapeutic applications. Thus, in a further embodiment the invention relates to a compound obtained or identified according to the method of the invention said compound being an agonist/activator of hypoxia inducible gene expression and/or function or an antagonist/inhibitor of hypoxia inducible gene expression and/or function. Given the therapeutic value of the compounds identified in accordance with the above-described method the present invention also relates to a method for the production of a pharmaceutical composition comprising the steps of the method of the invention and a step of formulating the so identified compound in a pharmaceutically acceptable form.

The therapeutically useful compounds identified according to the method of the invention may be administered to a patient by any appropriate method for the particular compound, e.g., orally, intravenously, parenterally, transdermally, transmucosally, or by surgery or implantation (e.g., with the compound being in the form of a solid or semi-solid biologically compatible and resorbable matrix) at or near the site where the effect of the compound is desired. For example, a salve or transdermal patch that can be directly applied to the skin so that a sufficient quantity of the compound is absorbed to increase vascularization locally may be used. This method would apply most generally to wounds on the skin. Salves containing the compound can be applied topically to induce new blood vessel formation locally, thereby improving oxygenation of the area and hastening wound healing. Therapeutic doses are determined to be appropriate by one skilled in the art.

Furthermore, identification of transacting factors which interact with the regulatory sequences of the invention can form the basis for the development of novel therapeutics for modulating hypoxic conditions associated with, for example ischemia, angiogenesis, vascular disease, and wound healing. Identification of transacting factors is carried out using standard methods in the art (see, e.g., Sambrook, supra, and Ausubel, supra) or methods as described in the appended examples. To determine whether a protein binds to the regulatory sequences of the invention, standard DNA footprinting and/or native gel-shift analyses can be carried

out. In order to identify a transacting factor which binds to the regulatory sequence of the invention, the regulatory sequence can be used as an affinity reagent in standard protein purification methods, or as a probe for screening an expression library. Once the transacting factor is identified, modulation of its binding to the regulatory sequences of the invention can be pursued, beginning with, for example, screening for inhibitors of transacting factor binding. Enhancement of hypoxia inducible expression in a patient, and thus enhancement of angiogenesis, may be achieved by administration of the transacting factor, or the gene encoding it (e.g., in a vector for gene therapy). In addition, if the active form of the transacting factor is a dimer, dominant-negative mutants of the transacting factor could be made in order to inhibit its activity. Furthermore, upon identification of the transacting factor, further components in the pathway of hypoxia signal transduction can be identified. Modulation of the activities of these components can then be pursued, in order to develop additional drugs and methods for modulating cell growth and angiogenesis.

Besides the identification of transacting factors it is also immediately evident to the person skilled in the art that antibodies can be raised against the compounds identified according to the method of the present invention. Thus, the present invention also relates to an antibody specifically recognizing the compound of the present invention.

Monoclonal antibodies can be prepared, for example, by the techniques as originally described in Köhler and Milstein, *Nature* 256 (1975), 495, and Galfré, *Meth. Enzymol.* 73 (1981), 3, which comprise the fusion of mouse myeloma cells to spleen cells derived from immunized mammals. Furthermore, antibodies or fragments thereof to the aforementioned MCPs or their receptors can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbour, 1988. These antibodies may be monoclonal antibodies, polyclonal antibodies or synthetic antibodies as well as fragments of antibodies, such as Fab, Fv, or scFv fragments etc.

Moreover the present invention relates to a pharmaceutical or diagnostic composition comprising the above-described compounds which are agonists/activators or

antagonists/inhibitors and/or antibodies and optionally a pharmaceutically acceptable carrier or suitable means for detection, respectively; see supra.

Further, the present invention relates to the use of the recombinant DNA molecule, vector, cell, pharmaceutical compositions, diagnostic compositions or a transgenic non-human animal of the invention for the identification of a chemical and/or biological substance capable of suppressing or activating and/or enhancing the transcription, expression and/or activity of hypoxia regulated genes and/or its expression products.

In a preferred embodiment, the chemical or biological substance used in the methods and uses of the present invention is selected from the group consisting of peptides, proteins, nucleic acids, antibodies, small organic compounds, hormones, neural transmitters, peptidomimics, and PNAs (Milner, Nature Medicine 1 (1995), 879-880; Hupp, Cell 83 (1995), 237-245; Gibbs, Cell 79 (1994), 193-198).

The present invention further relates to a method of inhibiting a vascular disease in a subject, comprising contacting an artery of said subject with the recombinant DNA molecule or vector of the invention, wherein said protein reduces or prevents the development of the vascular disease, and wherein preferably said protein reduces proliferation of smooth muscle cells.

In a further embodiment the present invention relates to the use of a recombinant DNA molecule, vector, nucleic acid molecule of the invention, compound and/or antibody of the invention for the preparation of a composition for directing and/or preventing expression of genes specifically during or after hypoxia induction and/or for the preparation of a pharmaceutical composition for treating, preventing and/or delaying a vascular disease, cardiac infarct or stroke, for ischemic preconditioning of organs and/or tissues, for enhancing angiogenesis, arteriogenesis, collateral growth of arteries and/or ischemic tolerance or for the stimulation of hypoxia function, and/or for treating, preventing and/or delaying a tumorous disease in a subject.

In a further embodiment, the present invention relates to the use of a recombinant DNA molecule, vector, nucleic acid molecule compound and/or antibody of the invention for the preparation of a pharmaceutical composition for inducing a vascular disease in a non-human animal or in a transgenic non-human animal described above.

In an additional preferred embodiment of the methods and uses of the invention, the vascular disease is selected from the group consisting of arteriosclerosis, coronary artery diseases, cerebral occlusive diseases, peripheral occlusive diseases, visceral occlusive diseases, renal artery diseases, mesenteric arterial insufficiency, ophthalmic and retinal occlusions and the tumorous disease is selected from the group consisting of colon carcinoma, sarcoma, carcinoma in breast, carcinoma in the head/neck, mesothelioma, glioblastoma, lymphoma and meningioma.

Further possible methods and uses in accordance with the present invention will be evident to the person skilled in the art and are described in, for example, WO 95/13387, WO 94/11499 and WO 97/00957.

The recombinant DNA molecules, vectors, nucleic acid molecules, compounds, uses and methods of the invention can be used for the treatment of all kinds of disorders and diseases hitherto unknown as being related to or dependent on hypoxia regulated genes or genes involved in ischemia genes. The recombinant DNA molecules, vectors, nucleic acid molecules, compounds, antibodies, methods and uses of the present invention may be desirably employed in humans, although animal treatment is also encompassed by the methods and uses described herein. Thus, the present invention provides for the use of a regulatory sequence as defined above for enhancing and/or directing hypoxia regulated gene expression in cells in any kind of eucaryotic organism.

These and other embodiments are disclosed and encompassed by the description and examples of the present invention. Further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on the

Internet, for example under <http://www.ncbi.nlm.nih.gov/PubMed/medline.html>. Further databases and addresses, such as <http://www.ncbi.nlm.nih.gov/>, <http://www.infobiogen.fr/>, http://www.fmi.ch/biology/research_tools.html, <http://www.tigr.org/>, are known to the person skilled in the art and can also be obtained using, e.g., <http://www.lycos.com>. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

Abbreviations: VEGF, Vascular Endothelial Growth Factor; hVEGF, human VEGF; EPO, Erythropoietin; HIF, Hypoxia-inducible factor; AP 1, Activator protein 1; ATF, Activating transcription factor; bZIP, basic leucine zipper; bHLH, basic helix-loop-helix; PAS, period-ARNT-singleminded; AhR, Arylhydrocarbon-Receptor; ARNT, AhR Nuclear Translocator; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; XRE, xenobiotic response element; aa, amino acid, UTR, untranslated region; VHL, von Hippel-Lindau; PNP, perinecrotic palisading.

The figures show:

Figure 1: (a) Alignments for mouseVEGF 3' untranslated region vs humanVEGF 3'-UTR.

(b) Alignment for mouseVEGF 3' untranslated region vs ratVEGF 3'-UTR.

Figure 2: LacZ reporter gene constructs used in the analysis of mechanisms upregulating VEGF expression *in vivo*. The length of the mouse VEGF 5' flanking region in the construct names is given relative to the transcription start site. mVEGF- mouse VEGF

Figure 3: Expression of the lacZ reporter gene in experimental GS9L gliomas. β -galactosidase histochemical staining is shown for tumors derived from GS9L cells stably transfected with the constructs P915 (a), P1130 (b),

PsvUTR (c) and P1130UTR (d). Sections were counterstained with neutral red. Necrosis - N. The bars represent 20mm.

Figure 4: *In situ* hybridization of a wild-type GS9L tumor.

Sections were hybridized with antisense riboprobes for VEGF (a, b) and HIF 1a (c-f). Brightfield and darkfield images of necrotic areas of the same sections are shown in a and b and c and d. A higher magnification of a non-necrotic area is shown in e and f. Control hybridizations with a sense probe for HIF 1a are shown in g and h. Arrowheads point to the palisading cells surrounding necrosis (N). T- viable tumor tissue. The bars represent 20mm.

Figure 5: β -galactosidase histochemical staining and EF 5 immunostaining of a tumor derived from P1130UTR transfected GS9L glioma cells. Serial sections were stained for expression of the lacZ reporter gene (a) and for the presence of hypoxic regions with an antibody directed against EF 5 (b). Necrosis - N. The bars represent 20mm.

Figure 6: Potentiating sequences for hypoxia induction of the human VEGF gene are present between bp -1176 and -1015. Deletion analysis of the human VEGF promoter was performed and the constructs were tested in transient transfection assays in C6 glioma cells. Transfection and hypoxic incubations were carried out as described in Example 3. The position of the human VEGF promoter fragments relative to the transcription initiation site is indicated on the left side. The -fold induction by hypoxia summarized on the right side represents the ratio of reporter gene activity obtained under hypoxic versus normoxic conditions. Each bar represents the mean value + SD of at least three independent transfections.

Figure 7: Potentiating sequences are not able to confer hypoxia responsiveness to a heterologous promoter. Transient transfection analysis of hVEGF enhancer fragments in combination with the SV40 promoter.

Transfection and hypoxic incubations were carried out as described in Example 3. The position of the human VEGF promoter fragments relative to the transcription initiation site is indicated on the left side. The -fold induction by hypoxia summarized on the right side represents the ratio of reporter gene activity obtained under hypoxic versus normoxic conditions. Each bar represents the mean value + SD of at least three independent transfections.

Figure 8: The potentiating effect of sequences between -1176 and -1015 on hypoxia induction is due an AP 1 consensus binding site. Site directed mutagenesis was performed on a luciferase fusion construct containing -1176 bp of human VEGF 5' flanking sequence to create internal deletions and point mutations of putative binding sites for the AhR-complex (Xenobiotic Response Element- XRE) or AP 1-transcription factors. The resulting expression vectors were tested in transient transfection assays in C6 glioma cells. Levels of induction by hypoxia are given relative to that obtained for the wildtype (-1176) construct. A sequence comparison for both the XRE and the AP 1 binding sites as well as the point mutations introduced are shown in b. Transfection and hypoxic incubations were carried out as described in Example 3. Each bar represents the mean value +SD of at least three independent transfections. EPO- Erythropoietin

Figure 9: Mutations interfering with the "potentiating" function prevent formation of a distinct DNA-protein-complex. Electrophoretic mobility shift assay was performed as described in Example 5. Nuclear extracts were prepared from hypoxic C6 cells. The AP 1 complex is indicated by an arrowhead. The point mutations introduced are shown on the right.

Figure 10: An AP 1 consensus binding site competes for DNA-protein-complex formation at the "potentiating" sequences. Competition analysis of the hVEGF oligonucleotide was performed as described in Example 6. Nuclear extracts were prepared from hypoxic C6 cells. The AP 1 complex

is indicated by an arrowhead.

Figure 11: The protein complex at the "potentiating" element consists of members of the Fos- and Jun-families of transcription factors. (a) Supershift analysis of the hVEGF oligonucleotide was performed as described in Experimental Procedures. Nuclear extracts were prepared from normoxic (N) or hypoxic (H) C6 cells. The AP 1 complex and supershifts are indicated by arrowheads. Use of oligonucleotides point-mutated in the AP 1 binding site (mutations see Fig. 9) demonstrates specificity of the Jun-supershift for the slowest migrating complex (b).

The examples illustrate the invention.

Example 1: Construction of reporter gene constructs comprising the 3'-UTR of the VEGF gene

To assess the possible contribution of the HIF 1 transcriptional enhancer site and the 3' mRNA stabilization element of the VEGF gene to the expression of VEGF in gliomas, four reporter gene constructs were produced that express β -gal (Fig. 2). Expression vectors were constructed (pSDK lacZ; Schlaeger, Development 121 (1995), 1089-1098) using a Kozak/Shine-Dalgarno/lacZ/ SV40-polyA-cassette, which contains a nuclear localization signal. 5' flanking sequences of the mouse VEGF gene (nucleotides 469 to 1247 of GenBank accession no. U41383) were obtained from pV5NBgl63, a subclone resulting from a screen of a λ FIXII genomic library (PCC4 teratocarcinoma cell line, Stratagene) with a mouse VEGF cDNA probe according to the standard methods described in Sambrook, supra. In addition, a 530bp fragment of genomic sequence, including the binding site for HIF 1, was amplified by PCR from 129SVJ mouse liver genomic DNA using the oligonucleotide primers 5'-CCTGGTGGGAGCTCTGGGCAG-3' (human sequence, SEQ ID NO. 3) and 5'-GACTTTGAG-CTCCCAAATAATTG-3' (SEQ ID NO. 4) and the Perkin-Elmer Ampli-Taq™ system, 1mM MgCl₂; 3' 94°C denaturation; 40 cycles; 1' 52°C annealing; 1' 72°C elongation; and thereafter 1' 94°C denaturation; 5' 52°C annealing; 10' 72°C

elongation. The cloned PCR product was reamplified with an upstream mouse primer (5'-CTGGCCTACCTACCTTTCTG-3'; SEQ ID NO. 5). The fragments obtained from the genomic clone and by PCR reamplification were fused using the common SacI site and cloned in front of the lacZ cassette. Deletion of the HIF 1 binding consensus sequence was obtained by BsaAI restriction digestion. For generation of lacZ constructs bearing the mouse VEGF 3'-UTR a 2.4kb HincII/ HindIII-fragment of a λ FIXII genomic clone was subcloned behind the lacZ gene. Fusion to the stop-codon was achieved by *in vitro* mutagenesis using the last 30bp of the lacZ gene and the first 137bp of the mouse VEGF 3'-UTR as the mutated oligomer: CATTACCAGTTGGTCTGGTGTCAAAAATAAGCCAGGCTGGCAGGAAG GAGCCTCCCTCAGGGTTTCGGGAACCAGACCTCTCACCGAAAGACCGATTAA CCATGTCACCACCACGCCATCATCGTCACCGTTGACAGAACAGTCCTTAATCCA GAAAGCCTGACAT (SEQ ID NO. 17).

Mutagenesis was performed using the M13 *in vitro* mutagenesis kit (BIORAD) according to the manufacturers instructions. The SV40 promoter was derived from the pGL2 promoter vector (Promega). All plasmid constructions were verified by dideoxy sequencing according to standard procedures known in the art. The reporter constructs P1130 and P915 were designed to investigate the role of the 5' regulatory region, and in particular the HIF 1 enhancer binding site. P1130 includes the consensus binding site for the Hypoxia-inducible factor 1, whereas P915 lacks this site but contains more proximal 5' sequences. Both of these constructs utilize the polyadenylation site from SV 40. Two other constructs addressed the contribution of the 3' untranslated region of VEGF. PsvUTR utilizes a generic 5' promoter region, derived from the SV40 early region, linked to the lac-Z gene, followed by the 3' untranslated region of the VEGF gene. P1130UTR utilizes the VEGF 5' regulatory region, including the HIF 1 site, along with the VEGF 3' region.

Example 2: Characterization of hypoxia mediated expression *in vitro* and *in vivo*

All four constructs described in Example 1 were transfected into GS9L cells, and multiple stable clones selected for each. GS9L glioma cells (Tom Budd, St.

Lawrence University New York State) were cultured in RPMI 1640 supplemented with 10% fetal calf serum. 5×10^6 cells were transfected by electroporation with 10 μ g of linearized lacZ reporter plasmid and 1 μ g of selection plasmid (pSV2Neo) containing the npt gene allowing the selection for G418 resistance. After 48 hours, cells were split into RPMI 1640/ 10% fetal calf serum containing 0.5mg/ml G418. Selection was performed on 96 well plates and arising clones were expanded. For genotyping cells were lysed in proteinase K/ PCR-lysis buffer (McMahon, Cell 62 (1990), 1073-1085) and PCR analysis was performed using the lacZ specific primers 5'-TCCTCTGCATGGTCAGGTC-3' (SEQ ID NO. 6) and 5'-CGTGGCCTGATTCATTCC-3' (SEQ ID NO. 7) and the Perkin Elmer System (s. above), 1.5mM $MgCl_2$; 3' 95°C denaturation; 30 cycles; 30" 95°C denaturation; 30" 58°C annealing; 45" 72°C elongation; and thereafter 1' 95°C denaturation; 2' 72°C elongation. An *in vitro* expression analysis of the selected clones was performed under normoxic and hypoxic (1% O_2 ; 5% CO_2 , 94% N_2 for 18 hours in a Forma Scientific/Labotect culture system (Model 3015)) conditions. Cell clones expressing the reporter gene under normoxic conditions or not expressing the reporter gene under hypoxic conditions were omitted from further analysis.

Clones showing hypoxia-inducible expression of β -gal were inoculated into syngeneic rats and the resultant tumors analyzed histologically for reporter gene expression. Subcutaneous transplantation into syngeneic Fischer 344 rats, excision of tumors and embedding were performed as described (Plate, Cancer Res. 53 (1993), 5822-5827). For detection of β -galactosidase activity, 10mm cryostat sections of the tumors were fixed in 2% paraformaldehyde in PIPES-buffer (0.1M PIPES, 0.5M $MgCl_2$, 0.2M EGTA, pH 6.9) for 5 minutes at room temperature. Staining was performed overnight at 37°C in 10mM each potassium ferrocyanide/ ferricyanide 1mM $MgCl_2$, 0.02% X-gal in PBS. Sections were counterstained with neutral red. Controls included staining of sections with neutral red only and incubation of GS9L wildtype tumor sections with X-Gal. Immunofluorescence for EF 5 was performed essentially as described by Waleh (Waleh, Cancer Res. 55 (1995), 6222-6226). Cell clones preselected for inducible reporter gene expression *in vitro* were found to show regulated expression *in vivo* as assessed by β -galactosidase staining on sections. For detection of regions of low oxygen partial pressure in the tumors 1% of the rat body mass EF 5 (Waleh, Cancer Res. 55 (1995), 6222-6226)

was given intravenously as a 10mM solution in PBS. Three hours after injection the animal was narcotized, the tumor was removed, immediately cooled in cold PBS and embedded in Tissue-Tek (Miles Scientific). EF 5 and the corresponding antibody can be obtained from Dr. Cameron J. Koch, Department of Radiation Oncology, University of Pennsylvania, Philadelphia. The analysis of tumors derived from P1130 transfected cells (Table 1, Fig. 3b) revealed weak β -gal expression only in PNP cells, indicating that this part of the VEGF 5' flanking region is sufficient to drive the expression of the reporter gene to this distinct cell population, albeit at a low level.

Table 1: Association of reporter based β -galactosidase staining with perinecrotic palisading (PNP) cells in experimental GS9L gliomas. Staining of palisading cells was visually evaluated over the necrotic areas of several sections. the overall appearance of the staining of palisading cells is indicated by - (no staining); + (weak staining, only part of the PNP cells stained); ++ (strong staining of many PNP cells) and +++ (intense staining of all PNP cells).

Construct	Clone	No. of animals	No. of necroses-bearing tumours	Staining of PNP cells
P1130	#2	2	1	+
	#6	4	2	+
	#8	2	0	+
P915	#8	2	1	-
	#11	2	1	-
	#14	4	3	-
PsvUTR	#3	2	2	++
	#5	2	1	-
	#7	4	4	++
P1130UTR	#6	4	1	+++
	#12	2	2	+++

In contrast, no β -galactosidase staining was detectable in sections of tumors derived from P915 transfected cells (Table 1, Fig. 3a). Since the binding site for HIF 1 was absent in this construct, the observation argues for an involvement of HIF 1 binding in the transcriptional activation of VEGF gene expression in a solid tumor *in vivo*.

At next the expression of the HIF 1 α mRNA was investigated by *in situ* hybridization.

In situ hybridization of the sections with a cRNA probe for VEGF164 was performed after fixation in 4% paraformaldehyde as described (Breier, Development 114 (1992), 521-532). The cRNA probe for mouse HIF 1 α was obtained by reverse transcription PCR on cytoplasmic RNA prepared from L929 cells which had been exposed to hypoxic conditions for 2 hours. Cytoplasmic RNA was prepared according to

Sambrook, reverse transcription using super-script™ reverse transcriptase (Life Technologies) and oligo dT₁₇X-primers was performed according to the instructions of the manufacturer. A 591bp fragment containing the PAS A and B domains of the protein was amplified using the degenerate primers PAS B3' (5'-KGTGGTSACTTGTCCTT-3'; SEQ ID NO. 8) and PAS A 5' (5'-GATGGYRAMATGATYTACAT-3'; SEQ ID NO. 9) deduced from the human sequence (Wang, Proc. Natl. Acad. Sci. USA 92 (1995), 5510-5514).

In Fig. 4d a strong signal for HIF 1 α mRNA is seen in the PNP cells, similarly to VEGF mRNA (arrowheads in Fig. 4b and d). However, intense hybridization signals for HIF 1 α mRNA were also detected in other parts of the tumor (Fig. 4e and f). This observation of relatively uniform expression of HIF 1 α mRNA is in accordance with the finding of Wenger et al., who did not find any variation of HIF 1 α mRNA expression levels in response to hypoxia *in vitro* (Wenger, Kidney Int. (1997), 560-563). Collectively their *in vitro* and our *in vivo* analyses suggest an additional level of regulation of HIF 1 activity. Recently, Huang et al. reported that HIF 1 activity depends on the stabilization of its α subunit (Huang, J. Biol. Chem., 271 (1996), 32253-32259).

The weak β -galactosidase staining observed in the P1130 tumors is in accordance with the moderate transcriptional activation of VEGF gene expression observed in response to hypoxia in C6 glioma cells *in vitro* (Ikeda, J. Biol. Chem. 270 (1995), 19761-19766). This result is, however, in clear contrast to the high level of VEGF mRNA detected in PNP cells of solid tumors. Thus additional mechanisms are implicated in the upregulation of VEGF gene expression in the PNP cells of gliomas. Based on *in vitro* indications, we tested the possible contribution of the 3' untranslated region of the VEGF gene to PNP cell-specific expression in gliomas, using the GS9L cells stably transfected with PsvUTR and P1130UTR. Sections of tumors derived from PsvUTR transfected cell clones showed an intense β -galactosidase staining of PNP cells, resembling the pattern found for VEGF mRNA in *in situ* hybridizations (Table 1, Fig. 3c and 4b), indicating that the 3'-UTR contains regulatory sequences that elicit expression in PNP cells. Even higher levels of β -gal, as assessed by X-gal staining of tissue sections, was observed in PNP-cells of tumors derived from P1130UTR transfected cell clones (Table 1, Fig. 3d); this reporter includes both 5' flanking and 3' untranslated regions of the VEGF gene.

These observations suggest that major regulatory sequences for VEGF gene expression in PNP cells *in vivo* are located in the 3'-UTR. Levy et al. (Levy, J. Biol. Chem. 271 (1996), 2746-2753; Levy, J. Biol. Chem. 271 (1996), 25492-25497) as well as Shima et al. (Shima, J. Biol. Chem. 271 (1996), 3877-3883) have reported the involvement of VEGF 3' untranslated sequences in the regulation of RNA stability under hypoxic conditions *in vitro*. Thus the strong PNP cell-specific expression of the 3'-UTR lacZ fusion constructs in experimental GS9L gliomas implies a major contribution of mRNA stabilization to the upregulation of VEGF gene expression in this tumor type.

The result that the combination of 3' untranslated sequences and the 5' flanking control region (P1130UTR) leads to a further increase in the expression of the reporter gene when compared to PsvUTR or P1130 tumors suggests cooperativity in the regulatory mechanisms governing the increase in VEGF expression in PNP cells of gliomas. This combination of regulatory mechanisms would facilitate the rapid response to minor changes in the oxygen partial pressure by HIF 1 mediated transcriptional activation (Jiang, Am. J. Physiol. 271 (Cell Physiol. 40) (1996), C1172-C1180) followed by the production of large amounts of VEGF from stabilized mRNA under longer lasting hypoxia (Ikeda, J. Biol. Chem. 270 (1995), 19761-19766). EF 5 immunofluorescence specific for hypoxic regions overlapped with β -galactosidase staining on P1130UTR derived tumors (Fig. 5). This finding in conjunction with the result that *in vitro* hypoxia-inducible mechanisms elicit VEGF upregulation in PNP cells of gliomas *in vivo* suggests that hypoxia is indeed a major microenvironmental stimulus leading to increased expression of VEGF in this cell type.

Example 3: Deletional analysis of the human VEGF 5' hypoxia regulatory element

As published previously (Ikeda, J. Biol. Chem. 270 (1995), 19761-19766) the *cis*-acting elements responsible for hypoxic activation of VEGF gene transcription in C6 glioma cells reside between bp -1176 and -888 in the human VEGF 5' flanking region. Meanwhile, the binding site for the Hypoxia-inducible factor 1 included in this fragment was reported to confer hypoxia activation to the human VEGF gene in

endothelial (Liu, Circ. Res. 77 (1995), 638-643) and Hep3B (Forsythe, Mol. Cell. Biol. 16 (1996), 4604-4613) cells, and to the rat VEGF gene in PC 12 cells (Levy, J. Biol. Chem. 270 (1995), 13333-13340). To test the hypothesis that sequences other than the HIF 1 consensus binding site contribute to the hypoxic response in C6 cells, further deletional analysis of sequences between -1176 and -888 was performed. All deletion constructs and constructs carrying mutations were derived from the human VEGF KpnI-NheI-luciferase fusion construct previously described (Ikeda, J. Biol. Chem. 270 (1995), 19761-19766). 5'-deletions to -1176, -1015, -973, and -888 were obtained by restriction enzyme digestion with SacI, PvuII, BsaAI, and BanI, respectively. *In vitro* mutagenesis for generation of internal mutations and deletions was carried out using the M13 *In vitro* Mutagenesis Kit (Biorad) according to the manufacturers instructions. C6 glioma cells (ATCC CCL 107) were propagated in Dulbecco's modified Eagles' medium (DMEM) supplemented with 10% fetal calf serum (PAN-Systems). Transfections, hypoxic incubations and analysis of cell lysates were carried out as previously described (Ikeda, J. Biol. Chem. 270 (1995), 19761-19766). Statistical calculations were carried out with the InStat 2.01 program. Compared to the hypoxic response of the -1176 construct, hypoxia induction was significantly decreased ($p < 0.001$) by shortening to bp -1015 (PvuII) (Fig. 6), although the HIF 1 binding consensus remained intact. Induction of the reporter gene expression was completely abolished when the HIF 1 site was destroyed (-973; BsaAI) (Fig. 6).

Example 4: Activation of a heterologous promoter

To test whether the sequences referred to above are able to activate a heterologous promoter in response to hypoxia, enhancer fragments comprising bp -1168 to -1015; -1015 to -888, and -1176 to -888 were subcloned and tested in front of the (non hypoxia responsive) SV40 promoter. To test the hypoxia induction of the subfragments -1176/-888; -1168/-1015 and -1015/-888 these were subcloned in front of the minimal SV40 promoter of the pGL2 promoter vector (Promega). Transfection experiments were performed as described in Example 3. In agreement with the results obtained with the homologous human VEGF promoter the -1015/-888 fragment containing the HIF 1 binding site showed a significantly ($p < 0.05$) diminished

hypoxic response compared to that of the -1176/-888 fragment. Surprisingly, the fragment potentiating the HIF 1 mediated effect (-1168/-1015) was not able to confer hypoxia responsiveness to the SV40 promoter on its own (Fig. 7).

Example 5: Mutational analysis of conserved sequences

To further investigate the role of upstream sequences in hypoxia induction, the HIF 1 binding site was deleted in the context of the fully responsive -1176 construct by site directed mutagenesis. This internal deletion completely abolished hypoxia induction (Fig. 6, -1176 Δ HIF), thus further supporting the finding that upstream sequences by themselves are unable to confer hypoxia responsiveness. This observation was made regardless of whether the homologous VEGF or the SV40 promoter were used. Addition of upstream sequences between -1176 and -1015 restored full inducibility. Database searches revealed a 6 bp element homologous to bp -77 to -72 of the human EPO promoter (GenBank accession no. M11319) in the region investigated. With the sequence TCACGC, this homology resembles a consensus Xenobiotic Response Element (XRE) reported to bind the AhR/Arnt/TCDD-complex (Whitelaw, Mol. Cell. Biol. 13 (1993), 2504-2514). As the AhR and HIF 1 α share structural features, such as the PAS domains and the dimerization partner Arnt (Wang, Proc. Natl. Acad. Sci. USA 92 (1995), 5510-5514), it appeared possible that another PAS domain protein complex plays an accessory role in hypoxia activation. Competition of the two PAS domain proteins AhR and HIF 1 α for Arnt recruitment has recently been reported, although activation of the AhR pathway was shown to have no influence on HIF 1 mediated hypoxic induction (Gradin, Mol. Cell. Biol. 16 (1996), 5221-5231). In agreement with this finding, our transfection studies with a reporter gene construct lacking the XRE (-1122 to -1116), revealed no change in hypoxic induction (Fig. 8 Δ XRE) suggesting that the AhR-complex is not involved in transcriptional activation by hypoxia. A search of the database revealed a CACACAG sequence-stretch (bp -921 to -912) with high homology to an element in the human EPO 3' enhancer. Neither replacement with the mouse EPO sequence nor internal deletion of the motif had an effect on the hypoxia induction of the human VEGF luciferase fusion construct.

To address the question of which *cis*-acting element(s) are involved in the

potentiation of HIF 1 mediated hypoxic induction, internal deletions and point mutations were introduced between bp -1176 and -1015. Comparison of analogous sequences of the human and mouse VEGF promoters revealed several conserved stretches of nucleotides. One of these (-1131/-1123) revealed an 8 out of 9 bp homology to one of the recently postulated bZIP/ATF binding sites in the promoter of the anoxia inducible VL30 retrotransposon (Estes, Exp. Cell Res. 220 (1995), 47-54). Deletion of this element in the -1176 construct (Fig. 8, DAP 1) significantly diminished ($p=0.001$) the hypoxia induction to the level observed with the -1015 deletion mutant (Fig. 8). The same observation was made using point mutations of the consensus site (Fig. 8, AP1M1 and AP1M2) previously described to prevent AP 1 binding (Schüle, Cell 61 (1990), 497-504). These results suggest that the AP 1 consensus binding site is indeed the element involved in hypoxia induced gene expression.

Example 6: Transcription Factor binding to the regulatory sequence

To determine which transcription factor(s) binding to the *cis*-element identified, electrophoretic mobility shift assays were performed. Nuclear extracts of normoxic and hypoxic (18 hours, 1% oxygen) C6 cells were prepared according to Semenza and Wang (Semenza, Mol. Cell. Biol. 12 (1992), 5447-5454). Annealing, purification and labeling of the oligonucleotides was performed as described (Leibiger, Biol. Chem. Hoppe-Seyler 375 (1994), 93-98). Incubation of the double-stranded oligonucleotides hVEGF 5'-TGGCGGGTAGGTTTGAATCATCACGCAGGC-3' (SEQ ID NO.10), hVEGF 5' DEL 5'-TGGCGGGTAGGTCACGCAGGC-3' (SEQ ID NO. 11), AP1M1 5'-TGGCGGGTAGGTTAGAATCATCACGCAGGC-3' (SEQ ID NO. 12) or AP1M2 5'-TGGCGGGTAGGTTTGGTTCATCACGCAGGC-3' (SEQ ID NO. 13) with 4µg of nuclear extracts was performed in the presence of 10mM Hepes (pH 7.9), 200mM NaCl, 4% Ficoll, 4mM DTT, 1mM EDTA, 0.1mg/ml BSA and 1mg poly (dl-dC) for 20 minutes at room temperature. The unlabeled competitor oligonucleotides AP.1 comp 5'-CGCTTGATGACTCAGCCGGAA-3' (SEQ ID NO. 14), ATF comp 5'-AGAGATTGCCTGACGTCAGAGAGCTAG-3' (SEQ ID NO. 15) or VL30 5'-TTTGAATGAGCCAATTGTA-3' (SEQ ID NO. 16) (Estes, Exp. Cell Res. 220 (1995), 47-54) were added at a 50-fold molar excess 10 minutes prior to the addition

of the labeled probe. For supershift analysis 1 μ g of the respective antibodies was added to the completed reaction mixture and incubation was performed for 2 hours at 4°C. Antibodies directed against Jun-family members (D), Fos-family members (K25), ATF-4 (Z5) and ATF-1 (25C10G) were obtained from Santa Cruz Biotechnology.

Incubation of a double-stranded oligonucleotide spanning the putative AP 1 binding site with nuclear extracts prepared from normoxic or hypoxic C6 cells did not reveal any differences in the binding pattern and affinity of the DNA-protein complexes (Fig. 11, lanes 1 and 2). Point mutations (Fig. 9, lanes 2 and 3) or an internal deletion (Fig. 9, lane 1) introduced into the oligonucleotide prevented the formation of the slowest migrating complex. In combination with the functional data this finding suggests that this complex mediates the potentiating effect. Three lines of evidence indicate that the proteins constituting the slowest migrating complex formed with hypoxic nuclear extracts belong to the Fos- and Jun-families of transcription factors: (1) Specific competition for this complex was observed with a 50-fold molar excess of an unlabeled oligonucleotide containing an AP-1 consensus binding site (Fig. 10, lane 6), and less efficient competition using an ATF consensus site (Fig. 10, lane 5/ complete competition at 100-fold molar excess, data not shown). (2) No complex formation was observed with point mutations known to prevent AP 1 binding (Schüle, Cell 61 (1990), 497-504) (Fig. 9, lanes 2 and 3). (3) Supershift analysis revealed immunoreactivity for Fos- and Jun-family members in this complex (Fig. 11a, lanes 5 and 6). The specificity of the partial Jun-supershift for this complex was proven by using the mutated oligonucleotides in the analysis: Prevention of formation of the slowest migrating complex did equally prevent formation of the supershift. The faster migrating complexes are unaffected (Fig. 11b). Similar results were obtained by using nuclear extracts prepared from normoxic cells. Finally, no competition was observed for the VL 30 oligonucleotide known to bind ATF-4 in anoxic fibroblasts (Fig. 10, lane 4) and antibodies against ATF-4 and ATF-1 did not supershift the complex (Fig. 11, lanes 3 and 4).

In the assays immunoreactivity was detected for both Fos- and Jun-family members, indicating that the transcription factor is composed of a Fos-Jun-heterodimer. Unexpectedly, the α Jun-antibody supershifted only parts of the complex. A lower

affinity of the α Jun-antibody to the rat Jun-protein present in the complex, when compared to the human c-jun it was raised against, would be one explanation for this observation. A more attractive model would be the steric hindrance of antibody binding to Jun by a third protein. As the epitope recognized by the antibody (aa247-263), and the binding interface for the coactivator p300 (up to aa246 for maximum binding, (Lee, Mol. Cell. Biol. 16 (1996), 4312-4326)), are immediately adjacent, steric hindrance by the presence of p300 appears possible. The coactivator p300 is known to provide physical links between enhancer bound transcription factors and the RNA polymerase II complex (Janknecht, Curr. Biol. 6 (1996), 951-954). Recently, it has been reported that p300 is part of the hypoxia-inducible HIF 1 complex (Arany, Proc. Natl. Acad. Sci. USA 93 (1996), 12969-12973). To date, the partner to which p300 bridges in this complex is unknown. Jun constitutively bound to an upstream sequence would be an attractive partner.

The present invention is not to be limited in scope by its specific embodiments described which are intended as single illustrations of individual aspects of the invention and any DNA molecules, or vectors which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those shown and described therein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Said modifications intended to fall within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Max-Planck-Gesellschaft zur Foerderung der
Wissenschaften, e.V.
(B) STREET: none
(C) CITY: Berlin
(D) STATE: none
(E) COUNTRY: Germany
(F) POSTAL CODE (ZIP): none

(ii) TITLE OF INVENTION: Regulatory sequences capable of regulating
hypoxia inducible expression in vivo

(iii) NUMBER OF SEQUENCES: 17

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2451 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TGAGCCAGGC TGGCAGGAAG GAGCCTCCCT CAGGGTTTCG GGAACCAGAC CTCTCACCGG	60
AAAGACCGAT TAACCATGTC ACCACCACGC CATCATCGTC ACCGTTGACA GAACAGTCCT	120
TAATCCAGAA AGCCTGACAT GAAGGAAGAG GAGACTCTTC GAGGAGCACT TTGGGTCCGG	180
AGGGCGAGAC TCCGGCAGAC GCATTCCCGG GCAGGTGACC AAGCACGGTC CCTCGTGGGA	240
CTGGATTTCG CATTCTCTTA TATCTGCTGC TAAATCGCCA AGCCCGGAAG ATTAGGGTTG	300
TTTCTGGGAT TCCTGTAGAC ACACCCACCC ACATACACAC ATATATATAT ATTATATATA	360
TAAATAAATA TATATGTTTT ATATATAAAA TATATATATA TTCTTTTTTT TAAATTAAC	420
CTGCTAATGT TATTGGTGTC TTCACTGGAT ATGTTTGACT GCTGTGGACT TGTGTTGGGA	480

GGAGGATGTC CTCACCTCGGA TGCCGACATG GGAGACAATG GGATGAAAGG CTCAGTGTG	540
GTCTGAGAGA GGCCGAAGTC CTTTGTGCTG CCGGGGAGCA AGCAAGGCCA GGGCACGGGG	600
GCACATTGGC TCACTTCCAG AAACACGACA AACCATTCC TGGCCCTGAG TCAAGAGGAC	660
AGAGAGACAG ATGATGACAG AGAAAGAGAT AAAGATGCCG GTTCCAACCA GAAGTTTGGG	720
GAGCCTCAGG ACATGGCATG CTTTGTGGAT CCCCATGATA GTCTACAAA GCACCCCGCC	780
CCTCTGGGCA CTGCCTGGA GAATCGGGAG CCTGGCCAGC CTTAGCTCG CTCCTCCACT	840
TCTGAGGGGC CTAGGAGGCC TCCACAGGT GTCCCGGCAA GAGAAGACAC GGTGGTGGAA	900
GAAGAGGCCT GGTAAATGGC CCTCCTCCTG GGACCCCTTC GTCCTCTCT TACCCACCT	960
CCTGGGTACA GCCCAGGAG ACCTTGTGTG ATCAGACCAT TGAAACCACT AATTCTGTCC	1020
CCAGGAGACT TGGCTGTGTG TGTGAGTGGC TTACCCTTCC TCATCTTCCC TTCCCAAGGC	1080
ACAGAGCAAT GGGGCAGGAC CCGCAAGCCC CTCACGGAGG CAGAGAAAAG AGAAAGTGTT	1140
TTATATACGG TACTTATTTA ATAGCCCTTT TTAATTAGAA ATTAACACAG TTAATTTAAT	1200
TAAAGAGTAG GGTTTTTTTC AGTATTCTTG GTTAATATTT AATTTCACCT ATTTATGAGA	1260
TGTATCTCTC GCTCTCTCTT ATTTGTACTT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT	1320
GTGTGTGTGT GTATGAAATC TGTGTTTCCA ATCTCTCTCT CCCAGATCGG TGACAGTCAC	1380
TAGCTTGTCC TGAGAAGATA TTTAATTTTG CTAACACTCA GCTCTGCCCT CCCTGTGCC	1440
CACCACACAT TCCTTTGAAA TAAGGTTTCA ATATACATTT ACATACTATA TATATATTTG	1500
GCAACTTGTG TTTGTATATA AATATATATA TATATATATG TTTATGTATA TATGTGATTC	1560
TGATAAAATA GACATTGCTA TTCTGTTTTT TATATGTAAA AACAAAACAA GAAAAATAGA	1620
GAATTCTACA TACTAAATCT CTCTCCTTTT TTAATTTTAA TATTGTATAT CATTTATTTA	1680
TTGGTGCTAC TGTTTATCCG TAATAATTGT GGGGGAAAAA GATATTAACA TCACGTCTTT	1740
GTCTCTAGAG CAGTTTCCG AGATATTCCG TAGTACATAT TTATTTTAA ACAGCAACAA	1800
AGAAATACAG ATATATCTTA AAAAAAAGC ATTTTGTATT AAAGAATTGA ATTCTGATCT	1860
CAAAGCTCTC CCTGGTCTCT CTTCTCTCC TGGGCCCTCC TGTCTCGCTT TCCCTCCTCC	1920
TTTGGGGTAC ATAGTTTTTG TCTTAGGTTT GAGAAGCAGT CCCTGGAGTA GAATATGGGG	1980
TGACCCATCC ATTCCTGGGC GGAGGGGAGA TGGCTCCTTT GCCAAGGGTC CTCACACTAC	2040
GTGTACTCT GTTCCTGTG AGACAAGGAT GGGGCCATGT CTCCAGGTGC TAACTGGAGA	2100
TCGGAGAGAG CTGTTGGCTG CAGCTGGCCA GGATTTGGCC ATGCCGGGGA CATGGGAGGC	2160
TGTGAGCCCA GCATGCAGTT TACTTCTGGG TGCTAAATGG AAGAGTCCAG TAAAAAGAGT	2220
CTTGCCCATG GGATTCCATT CCGCTTTGTG CAAGCCTTTC GGACCCATTC TTGTCCAGG	2280

CCACTCGGAC CCTGTTTGGA TCTTTGATAT AATAGCATTG TATCCAAATC TGCTTCTAGA 2340
ATCTTACTCC TCATGATAAC AAAGGGCCGG GGNAAACACA GGGCTGGCTA GTATGGTTCT 2400
AGTACCGATA GACTATGCCA CCTGCAAGAG CAGGGGAGGG GCACAAAGCT T 2451

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1167 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CTGGCCTACC TACCTTTCTG AATGCTAGGG TAGGTTTGAA TCACCATGCC GGCCTGGCCC 60
GCTTCTGCCC CCATTGGCAC CCTGGCTTCA GTTCCCTGGC AACATCTCTG TGTGTGTGTG 120
TGTGTGTGTG AGAGAGAGAG ATCAGGAGGA ACAAGGGCCT CTGTCTGCCC AGCTGTCTCT 180
CCTTCAGGGC TCTGCCAGAC TACACAGTGC ATACGTGGGT TTCCACAGGT CGTCTCACTC 240
CCCCGCACTG ACTAACTCCA GAACTCCACT TCCCGTTCTC AGTGCCACAA ATTTGGTGCC 300
AAATTCTCTC CAGAGAAGCC TCTCTGAAA CTTCCCAGAG GATCCCATTG ACCCCAGGGC 360
CCTAGCTCCT GATGACTGCA GATCAGACAA GGGCTCAGAT AAGCATACTC CCCCCCCCCC 420
GTAACCCCTT CCCCACATAT AAACCTAGAG TTATGCTTCC GAGGTCAAAC ACGCAACTTT 480
TTGGGTGTGT GTGTATGTCA GAAACACGCA ATTATTTGGG AGCTCAAAGT CTGCCGCACT 540
CAAGAATCAA CTCTCACCCC CTTTCCAAGA CCCGTGCCAT TTGAGCAAGA GTTGGGGTGT 600
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GCCGAGTCTG CGTGAGGGAG GACGCGTGTG TCAATGTGAG TCGGTGCATG CATGTGTGTG 720
TGTGTGTAGT GTGTTTGTGA GGTGGGGGAG AAAGCCAGGG GTCACTCTAG TTGTCCCTAT 780
CCTCATACGT CCCTGCCAGC TCTCCGCCTT CCAACCCCTA CTTTCTCCTA TATTCCTGGG 840
AAAGGGAATT GTTCTTAGAC CCTGTCCGCA TATAACCTCA CTCTCCTGTC TCCCCTGATT 900
CCCAATACTC TGGGATTCCC AGTGTGTTCG TGAGCCCAGT TTGAAGGGGT GCACAGATAA 960
TTTTGAGGCC GTGGACCCTG GTAAGGGGTT TAGCTTTCCA TTTCGCGGTA GTGGCCTAGG 1020
GGCTCCCCGA AAGGCGGTGC CTGGCTCCAC CAGACCGCTC CCCGGGGCGG GTCTGGGCGG 1080

GGCTTGGGGG TGGAGCTAGA TTTCCTCTTT TTCTTCCACC GCTGTTACCG GTGAGAAGCG 1140

CAGAGGCTTG GGGCAGCCGA GCTGCAG 1167

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCTGGTGGGA GCTCTGGGCA G 21

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GACTTTGAGC TCCCAAATAA TTG 23

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTGGCCTACC TACCTTTCTG

20

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

ATCCTCTGCA TGGTCAGGTC

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(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

CGTGGCCTGA TTCATTCC

18

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

KGTGGTSACT TGTCTT

17

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GATGGYRAMA TGATYTACAT

20

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TGGCGGGTAG GTTGAATCA TCACGCAGGC

30

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TGGCGGGTAG GTCACGCAGG C

21

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TGGCGGGTAG GTTAGAATCA TCACGCAGGC

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(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TGGCGGGTAG GTTTGGTTCA TCACGCAGGC

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(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CGCTTGATGA CTCAGCCGGA A

21

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

AGAGATTGCC TGACGTCAGA GAGCTAG

27

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TTTGAATGAG CCAATTGTA

19

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 167 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "oligonucleotide"

(iii) HYPOTHETICAL: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CATTACCAGT TGGTCTGGTG TCAAAAATAA GCCAGGCTGG CAGGAAGGAG CCTCCCTCAG	60
GGTTTCGGGA ACCAGACCTC TCACCGGAAA GACCGATTAA CCATGTCACC ACCACGCCAT	120
CATCGTCACC GTTGACAGAA CAGTCCTTAA TCCAGAAAGC CTGACAT	167

Claims

1. A recombinant DNA molecule comprising:
 - (a) a first regulatory sequence of a promoter active in mammalian cells;
 - (b) operatively linked thereto a heterologous DNA sequence; and
 - (c) operatively linked thereto a second regulatory sequence derived from the 3'-untranslated region of the Vascular Endothelial Growth factor (VEGF) gene or of a gene homologous to the VEGF gene being capable of regulating hypoxia inducible expression *in vivo*.
2. The recombinant DNA molecule of claim 1, wherein said second regulatory sequence is selected from the group consisting of
 - (a) DNA sequences comprising a nucleotide sequence as given in SEQ ID NO. 1;
 - (b) DNA sequences comprising the nucleotide sequence of the 3'-untranslated region from the human or rat VEGF gene;
 - (c) DNA sequences comprising a nucleotide sequence which hybridizes with a nucleotide sequence of (a) or (b) under stringent conditions and which is capable of regulating hypoxia induced expression *in vivo*;
 - (d) DNA sequences comprising a nucleotide sequence which is conserved in the nucleotide sequences of (a) and (b); and
 - (e) DNA sequences comprising a fragment, analogue or derivative of a nucleotide sequence of any one of (a) to (d) capable of regulating hypoxia inducible expression *in vivo*.
3. The recombinant DNA molecule of claim 1 or 2, wherein said first regulatory sequence comprises an AP-1 binding site, an SP1 binding site, a Hypoxia inducible Factor (HIF) 1 binding site or any combination(s) thereof.
4. The recombinant DNA molecule of claim 3, wherein said first regulatory sequence comprises an AP-1 and an HIF1 binding site.
5. The recombinant DNA molecule of any one of claims 1 to 4, wherein said first regulatory sequence is derived from a promoter of hypoxia inducible genes, genes encoding growth factors or its receptors or glycolytic enzymes.
6. The recombinant DNA molecule of claim 5, wherein said growth factor is VEGF, PDGF or Fibroblast growth factor.

7. The recombinant DNA molecule of any one of claims 1 to 6, wherein said first regulatory sequence comprises a minimal promoter.
8. The recombinant DNA molecule of claim 7, wherein said minimal promoter is derived from SV40.
9. The recombinant DNA molecule of any one of claims 1 to 8, wherein said first regulatory sequence comprises a DNA sequence selected from the group consisting of
 - (a) DNA sequences comprising the nucleotide sequence as given in SEQ ID NO. 2; preferably from nucleotide 1 to nucleotide 280;
 - (b) DNA sequences comprising the nucleotide sequence of the human VEGF promoter;
 - (c) DNA sequences comprising a nucleotide sequence which hybridizes with a nucleotide sequence of (a) or (b) under stringent conditions and which is capable of regulating hypoxia induced expression *in vivo*;
 - (d) DNA sequences comprising a nucleotide sequence which is conserved in the nucleotide sequences of (a) and (b); and
 - (e) DNA sequences comprising a fragment, analogue or derivative of a nucleotide sequence of any one of (a) to (d) capable of regulating hypoxia inducible gene expression.
10. The recombinant DNA molecule of any one of claims 1 to 9, wherein said heterologous DNA sequence encodes a peptide, protein, antisense RNA, sense RNA and/or ribozyme.
11. The recombinant DNA molecule of claim 10, wherein said protein is selected from the group consisting of VEGF, Hypoxia Inducible Factors (HIF), HIF-Related Factor (HRF), tissue plasminogen activator, p21 cell cycle inhibitor, nitric oxide synthase, interferon- γ , atrial natriuretic polypeptide, p53, proteins encoded by apoptosis inducing genes of the bcl2 family and monocyte chemotactic proteins.
12. The recombinant DNA molecule of claim 10, wherein said protein is a scorable marker, preferably luciferase, green fluorescent protein or lacZ.
13. The recombinant DNA molecule of claim 10, wherein said antisense RNA or said ribozyme are directed against a gene involved in vasculogenesis and/or angiogenesis and/or tumors.

14. A nucleic acid molecule of at least 15 nucleotides in length hybridizing specifically with the second regulatory sequence of the recombinant DNA molecule of any one of claims 1 to 13.
15. A vector comprising a recombinant DNA molecule of any one of claims 1 to 13.
16. The vector of claim 15, which is an expression vector and/or a targeting vector.
17. A cell transformed with a DNA molecule of any one of claims 1 to 13 or the vector of claim 15 or 16.
18. The cell of claim 17, which is a prokaryotic or eukaryotic cell, preferably a glioma cell, primary cell, tumor cell, spheroid cell, aggregate cell, stem cell or a differentiated cell.
19. A pharmaceutical composition comprising a recombinant DNA molecule of any one of claims 1 to 13, the vector of claim 15 or 16 and/or the nucleic acid molecule of claim 16 and optionally a pharmaceutically acceptable carrier.
20. A diagnostic composition comprising a recombinant DNA molecule of any one of claims 1 to 13, the vector of claim 15 or 16 and/or the nucleic acid molecule of claim 14, and optionally suitable means for detection.
21. A method for the production of a transgenic non-human animal, preferably mouse comprising introduction of the recombinant DNA molecule of any one of claims 1 to 13 or the vector of claims 15 or 16 into a germ cell, embryonic cell or an egg cell or a cell derived therefrom.
22. A transgenic non-human animal comprising stably integrated into its genome a recombinant DNA molecule of any one of claims 1 to 13 and/or the vector of claim 15 or 16 or obtained by the method of claim 21.
23. A method for the identification of an agonist/activator and antagonist/inhibitor of genes or gene products involved in hypoxia and/or ischemia comprising the steps of:
 - (a) providing an animal or human cell, or tissue or a non-human animal comprising a recombinant DNA molecule comprising a readout system operatively linked to at least one regulatory sequence capable of mediating

or regulating hypoxia and/or ischemia inducible expression of said readout system, wherein said regulatory sequence is preferably the first and/or second regulatory sequence as defined in any one of claims 1 to 9;

- (b) culturing said animal or human cell, or tissue or non-human animal in the presence of a sample comprising a plurality of compounds under conditions which permit inducible expression of said readout system;
- (c) identifying a sample and compound, respectively, which leads to suppression or activation and/or enhancement of expression of said readout system in said animal or human cell, or tissue or non-human animal.

24. The method of claim 23 further comprising the step of

- (d) identifying and/or isolating from the identified sample the compound responsible for said suppression or activation and/or enhancement of expression of said readout system in said animal or human cell, or tissue, or non-human animal.

25. The method of claim 23 or 24 further comprising the step of

- (e) determining whether said sample or compound mimics or suppresses the cellular effects of hypoxia.

26. A method for the production of a pharmaceutical composition comprising the steps of the method of any one of claims 23 to 25 and a step of formulating the compound identified in step (c), (d) or (e) in a pharmaceutically acceptable form.

27. The method of any one of claims 23 to 26, wherein said recombinant DNA molecule is a recombinant DNA molecule of any one of claims 1 to 13 or a vector of claim 16 or 17.

28. The method of any one of claims 23 to 27, wherein said cell is a cell of claim 17 or 18.

29. The method of any one of claims 23 to 28, wherein said non-human animal is a transgenic non-human animal of claim 22.

30. The method of any one of claims 23 to 29, wherein said DNA molecule is introduced into the genome by transfection, transformation, electroporation, infection or particle bombardment.

31. The method of any one of claims 23 to 30, wherein induction of hypoxia is performed by modulation of O₂ concentration, or conferred by a compound capable of mimicking effects of hypoxia, preferably cobaltous chloride, desferrioxamine, glucose and nutrient deprivation.
32. The method of claim 31, wherein said compound which is capable of inducing mimicking effects of hypoxia is a hypoxia-inducible factor (HIF) or an HIF-related-factor (HRF) or a nucleic acid molecule encoding either of said factors operatively linked to regulatory elements allowing expression of said nucleic acid molecules in said mammalian cell.
33. A compound obtained or identified by the method of any one of claims 23 to 32, which is an agonist/activator of hypoxia inducible gene expression and/or function.
34. A compound obtained or identified by the method of any one of claims 23 to 32, which is an antagonist/inhibitor of hypoxia inducible gene expression and/or function.
35. An antibody specifically recognizing the compound of claim 33 or 34.
36. A pharmaceutical composition comprising the agonist/activator of claim 33, the antagonist/inhibitor of claim 34 and/or the antibody of claim 35, and optionally a pharmaceutically acceptable carrier.
37. A diagnostic composition comprising the agonist/activator of claim 33, the antagonist/inhibitor of claim 34 and/or the antibody of claim 35, and optionally suitable means for detection.
38. Use of a recombinant DNA molecule of any one of claims 1 to 13, the vector of claim 15 or 16, the cell of claim 17 or 18, the pharmaceutical composition of claim 20, the diagnostic composition of claim 21 and/or the transgenic non-human animal of claim 22 for the identification of a chemical and/or biological compound capable of suppressing or activating and/or enhancing the transcription, expression and/or activity of hypoxia regulated genes and/or its expression products.

39. The method of any one of claims 23 to 32 or the use of claim 38, wherein the chemical and/or biological compound is selected from the group consisting of peptides, proteins, nucleic acids, antibodies, small organic compounds, hormones, neurotransmitters, peptidomimics and PNAs.
40. A method of inhibiting a vascular disease in a subject, comprising contacting an artery of said subject with the vector of claim 15 or 16, wherein said protein reduces or prevents the development of the vascular disease.
41. The method of claim 40, wherein said protein reduces or induces proliferation of vascular endothelial cells and smooth muscle cells.
42. Use of a recombinant DNA molecule of any one of claims 1 to 13, the vector of claim 15 or 16, the nucleic acid molecule of claim 14, the compound of claim 33 or 34 and/or the antibody of claim 35 for the preparation of a composition for directing or preventing expression of genes specifically during or after hypoxia induction.
43. Use of a recombinant DNA molecule of any one of claims 1 to 13, the vector of claim 15 or 16, the nucleic acid molecule of claim 14, the compound of claim 33 or 34 and/or the antibody of claim 35 for the preparation of a pharmaceutical composition for treating, preventing and/or delaying a vascular disease, cardiac infarct or stroke, for ischemic preconditioning of organs and/or tissues, for enhancing angiogenesis, arteriogenesis, collateral growth of arteries and/or ischemic tolerance or for the stimulation of hypoxia function, and/or for treating, preventing and/or delaying a tumorous disease in a subject.
44. Use of a recombinant DNA molecule of any one of claims 1 to 13, the vector of claim 15 or 16, the nucleic acid molecule of claim 14, the compound of claim 33 or 34 and/or the antibody of claim 35 for the preparation of a pharmaceutical composition for inducing a vascular disease in a non-human animal or in the transgenic non-human animal of claim 22.
45. The use of claim 43, wherein said vascular disease is selected from the group consisting of arteriosclerosis, coronary artery diseases, cerebral occlusive diseases, peripheral occlusive diseases, visceral occlusive diseases, renal artery diseases, mesenteric arterial insufficiency, ophthalmic and retinal occlusions, or wherein the tumorous disease is selected from the group

consisting of colon carcinoma, sarcoma, carcinoma in breast, carcinoma in the head/neck, mesothelioma, glioblastoma, lymphoma and meningeoma.

46. Use of a second and optionally first regulatory sequence as defined in any one of claims 1 to 9 for regulating gene expression.

1/14

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1 GAGAGAGAGAG GAGAGCTTCC TCAGGGTTTC GGGAGCCAGA TCCTGTACCA GGAAGACCTG ATACAGAGAG ATCCATACAG ATCCAGAGAG
121 CAGTCTCTTAA TCCAGAAACC TGAATGACAG GAGAGAGAGA CTCTCCAG AGCACTTTG GTCGCGAGG CGAGACTGCG GCGAGAGCAT TCCCGCGCGG GTGAGCCGAG AGCGTCCCTT
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Fig. 1a continued

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Fig. 1b

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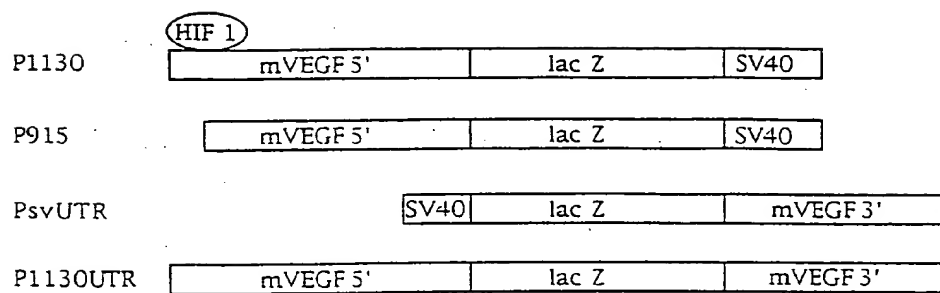


Fig. 2

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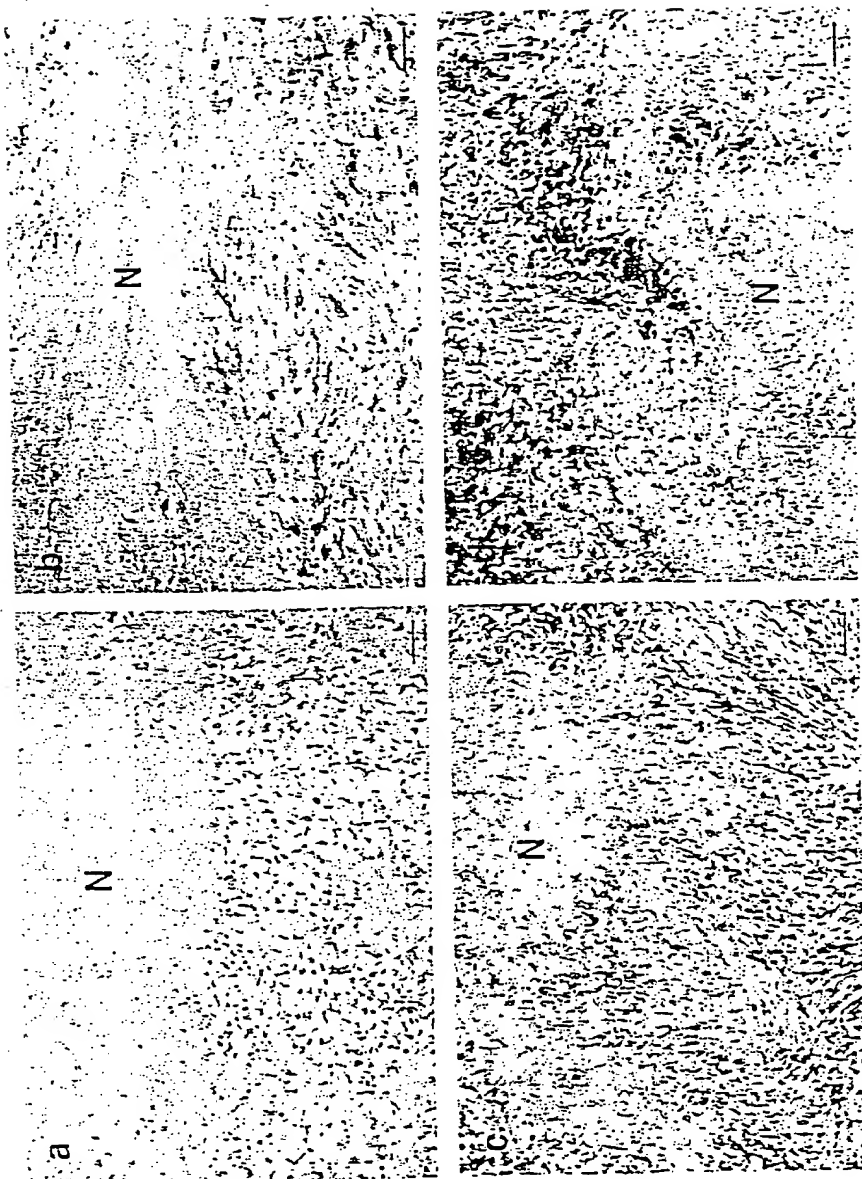


Fig. 3

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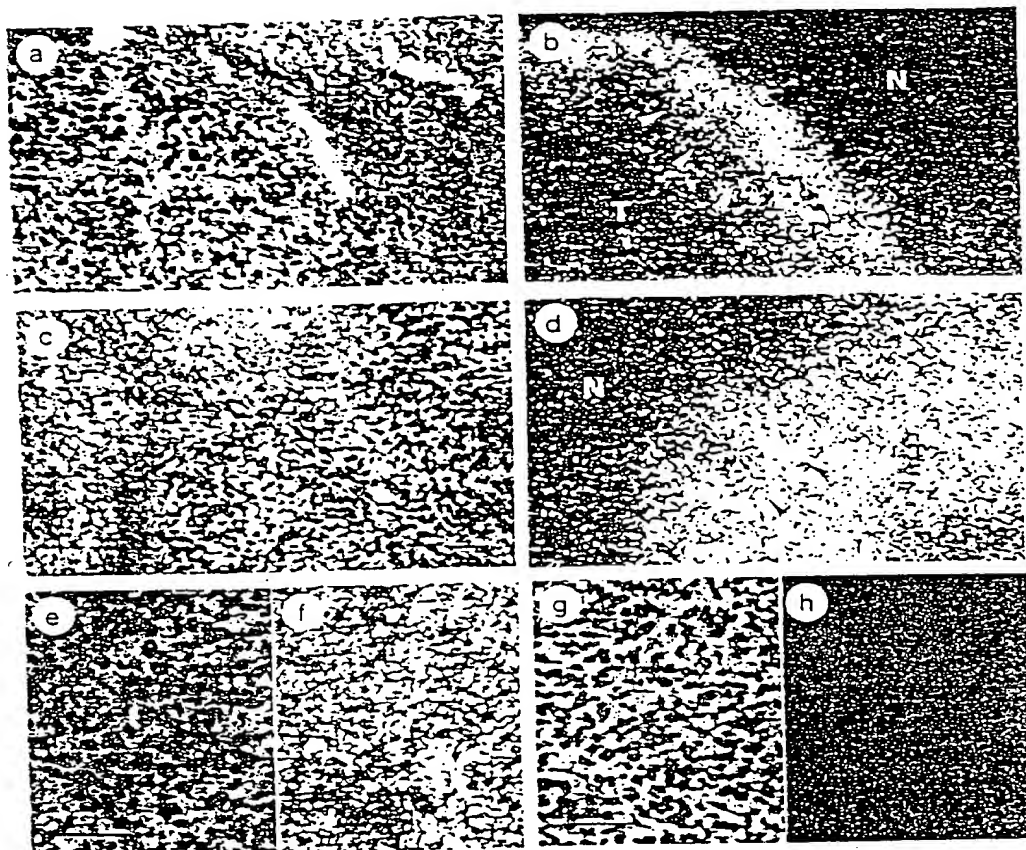


Fig. 4

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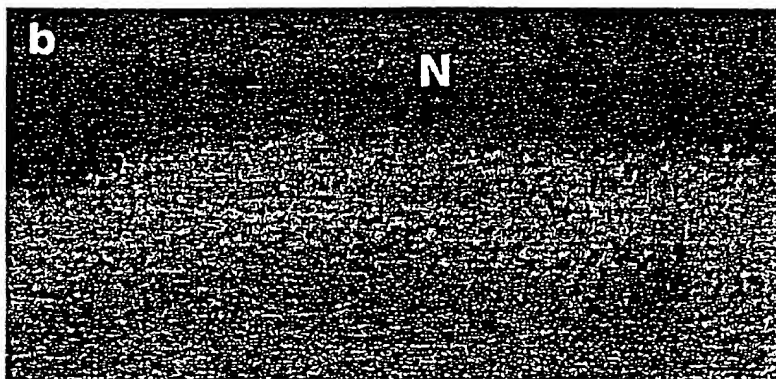
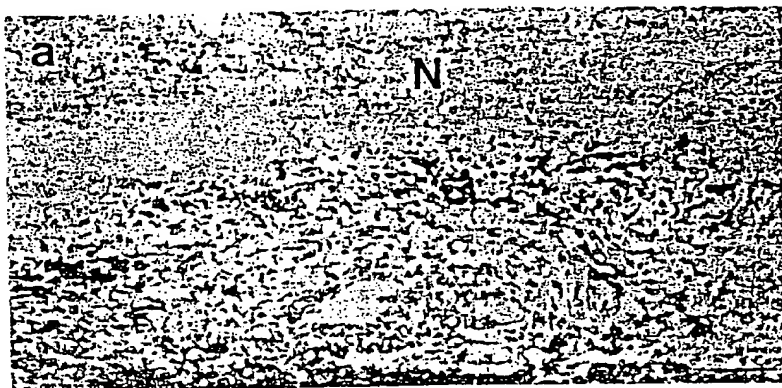


Fig. 5

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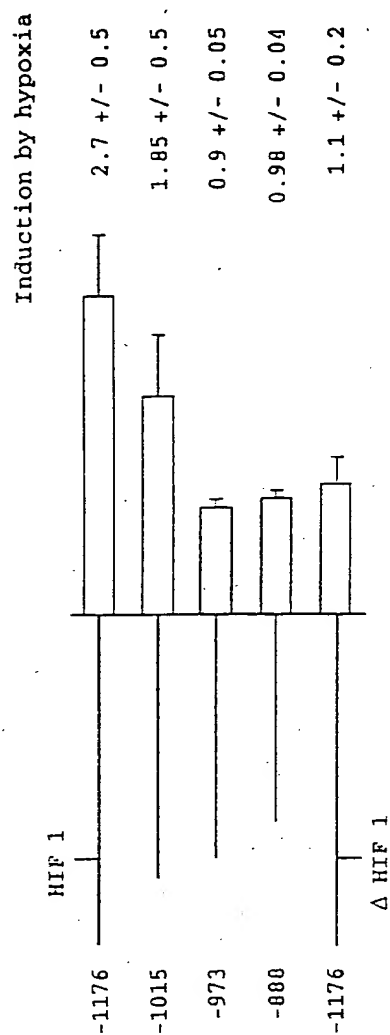


Fig. 6

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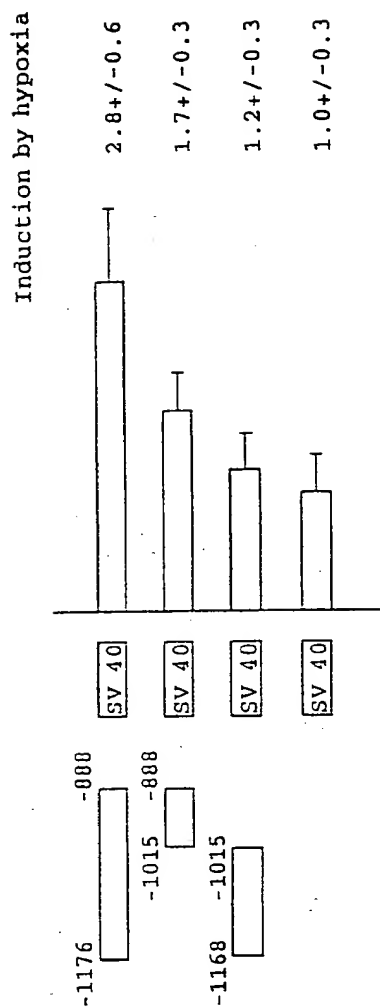


Fig. 7

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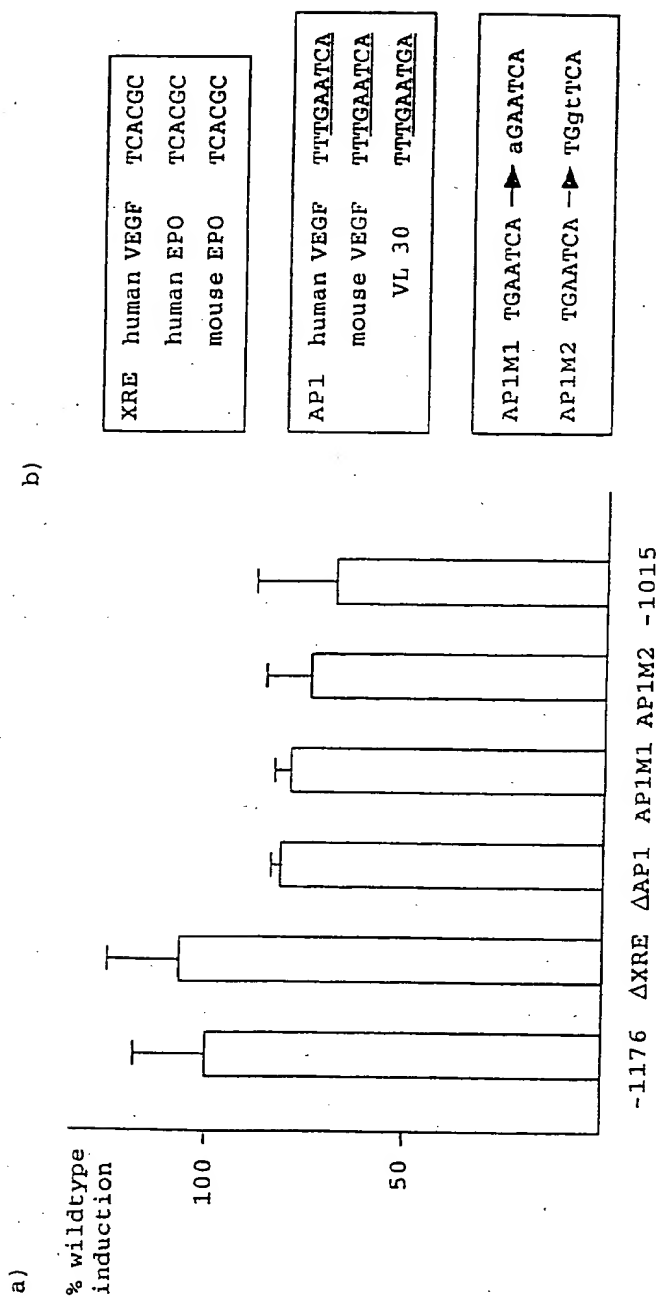


Fig. 8

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Probe hVEGF M1 M2 hVEGF
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M1	TGAATCA → aGAATCA
M2	TGAATCA → TGgtTCA
DEL	TGAATCA → Δ

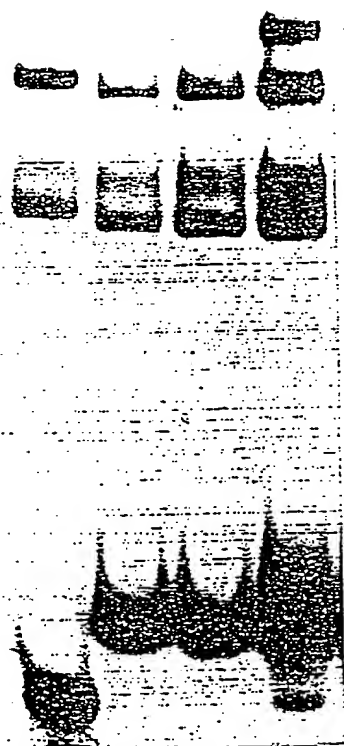


Fig. 9

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Probe	hVEGF				
Antibody	α ATF-4				
Competitor	-	-	hVEGF VL30	ATF comp	AP 1 comp

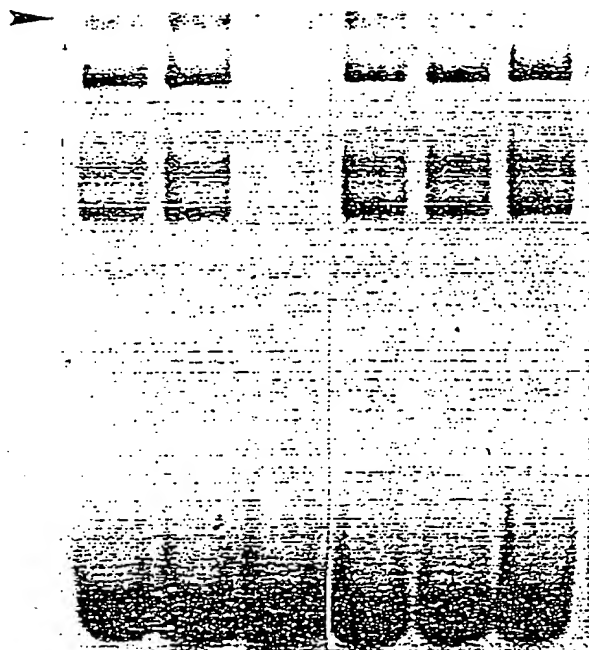


Fig. 10

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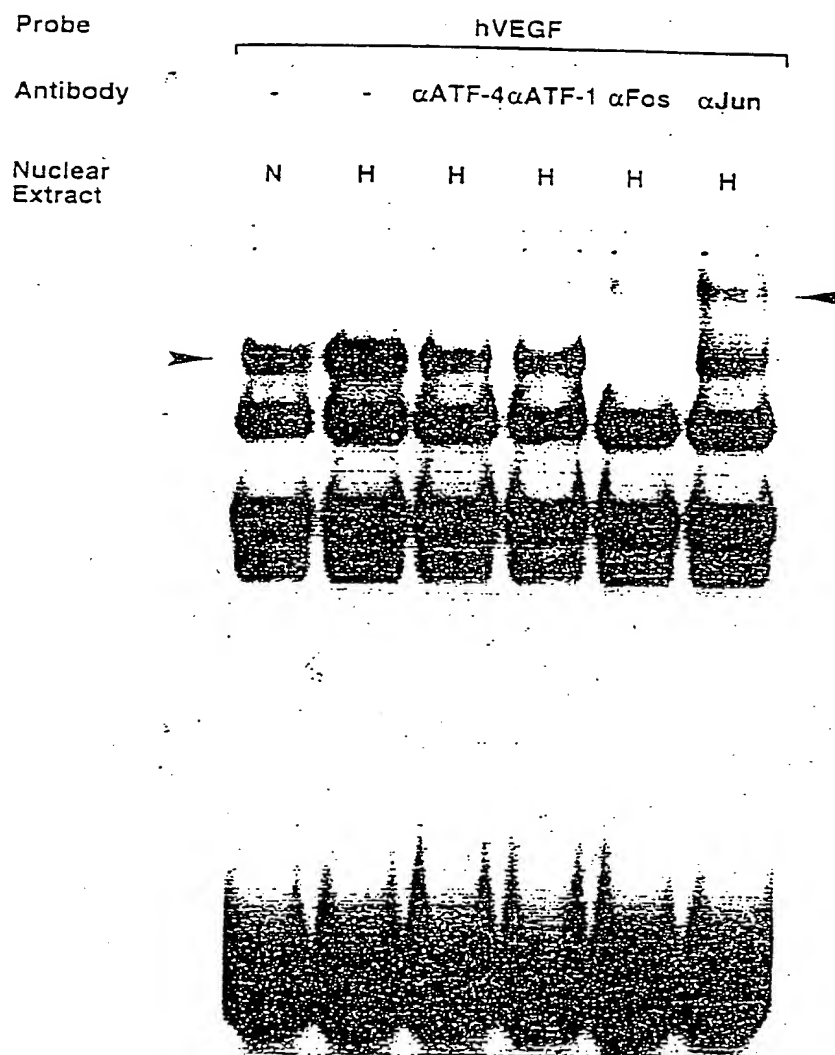
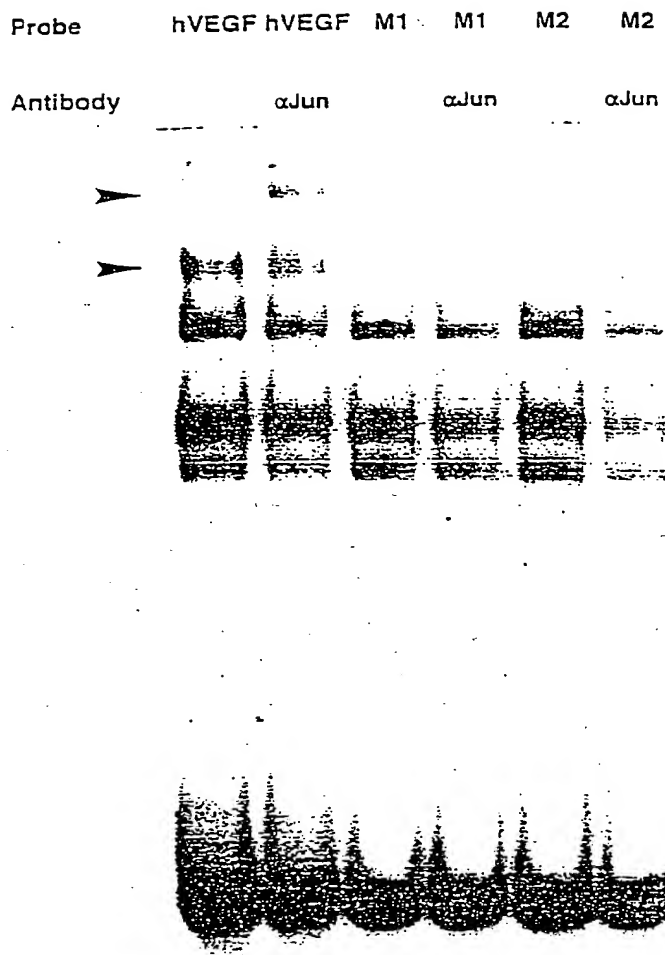


Fig. 11a

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 98/03517

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/85 C07K14/52 A01K67/027 A61K31/70 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SEMENZA G L ET AL: "A NUCLEAR FACTOR INDUCED BY HYPOXIA VIA DE NOVO PROTEIN SYNTHESIS BINDS TO THE HUMAN ERYTHROPOIETIN GENE ENHANCER AT A SITE REQUIRED FOR TRANSCRIPTIONAL ACTIVATION" MOLECULAR AND CELLULAR BIOLOGY, vol. 12, no. 12, 1 December 1992, pages 5447-5454, XP000562137 cited in the application	1, 2, 7, 8, 10, 12, 14-18, 23, 24, 27, 28, 30-33, 38, 39, 46
Y	see the whole document --- -/--	3-6, 11, 13, 19, 21

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "S" document member of the same patent family

Date of the actual completion of the international search

11 November 1998

Date of mailing of the international search report

27/11/1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel: (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Smalt, R

INTERNATIONAL SEARCH REPORT

Int. Patent Application No.

PCT/EP 98/03517

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 39426 A (UNIV JOHNS HOPKINS MED) 12 December 1996	1,7,8, 10,12, 14-18, 23-28, 31-40,46
Y	see the whole document, particularly claims 19,23-25, and examples 8 and 9.	3-6,9, 11,19, 21,42, 43,45
Y	LEVY, A.P. ET AL.: "Regulation of vascular endothelial growth factor by hypoxia and its modulation by the von Hippel-Lindau tumor suppressor gene." KIDNEY INTERNATIONAL, vol. 51, no. 2, February 1997, pages 575-8, XP002083931 see the whole document	9,27-29, 38,39
Y	WO 96 27006 A (HYBRIDON INC) 6 September 1996	13,42, 43,45
A	see the whole document	10
X	SHIMA, D.T. ET AL.: "The mouse gene for vascular endothelial growth factor." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 7, 16 February 1996, pages 3877-83, XP002083932 cited in the application see page 3878, right-hand column, paragraph 2 - paragraph 4: figures 6,8	1,2,7, 10,14, 15,17, 18,46
X	US 5 580 722 A (LIECHTFRIED FRANZ E ET AL) 3 December 1996	23-26,30
Y	see column 22, line 63 - line 67; figure 20 see column 34, line 1 - line 50 see column 43, line 34 - line 66	27-29, 38,39
A	MICHENKO, A. ET AL.: "Hypoxia regulatory elements of the human vascular endothelial growth factor gene." CELLULAR AND MOLECULAR BIOLOGY RESEARCH, vol. 40, no. 1, 1994, pages 35-9, XP002083933 see the whole document	
A	LEVY, A.P. ET AL.: "Transcription regulation of the rat vascular endothelial growth factor gene by hypoxia." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 22, 2 June 1995, pages 13333-40, XP002083934 cited in the application see the whole document	

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INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 98/03517

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	IKEDA, E. ET AL.: "Hypoxia-induced transcriptional activation and increased mRNA stability of vascular endothelial growth factor in C6 glioma cells." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 34, 25 August 1995, pages 19761-6, XP002083935 cited in the application see the whole document -----	
P,X	DAMERT A ; IKEDA E; RISAU W: "Activator-protein-1 binding potentiates the hypoxia-inducible factor-1-mediated hypoxia-induced transcriptional activation of vascular-endothelial growth factor expression in C6 glioma cells." BIOCHEMICAL JOURNAL, vol. 327, no. Pt 2, 15 October 1997, pages 419-23, XP002083936 see the whole document -----	23-25, 30-33,39

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 98/03517

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 40 and 41
is(are) directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☒ Claims Nos.: 33, 34, 36, 37, 42-45
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
These claims could not be searched to completion due to insufficient
characterization of the compounds of claims 33 and 34.
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 98/03517

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9639426 A	12-12-1996	AU 6332696 A CA 2222279 A EP 0833840 A	24-12-1996 12-12-1996 08-04-1998
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